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The goal of this investigation is to determine the role of accessory proteins as mediators of estrogen receptor (ER) activity both before and after ER interaction with specific DNA binding sites (EREs) on responsive genes. We wish to test the hypothesis that estrogens and estrogen antagonists promote altered ER-accessory protein interactions that lead to differential transcriptional activity. During the past year, we have characterized (by gel retardation analysis, filter binding assays, and DNA bending experiments) ER-associated proteins isolated by specific DNA (ERE) affinity chromatography, steroid affinity chromatography, and immunoadsorption from CHO-ER and MCF-7 breast cancer cell extracts. A major effect of of four such proteins (p45, p48, p55, and hsp70) in *in vitro* ER-ERE reconstitution experiments is to facilitate maximal ER-ERE interaction (capacity). Second, these proteins appear to contribute to the bend angle induced by ER interaction with EREs, which in turn may affect the rate of transcription initiation of responsive genes. We have also used a GST-ER ligand binding domain (LBD) fusion protein to identify additional proteins in mammalian cell extracts that bind to or modulate ER selectively in the presence of estrogen agonists or antagonists. At least one of these proteins phosphorylates ER-LBD in an *in vitro* kinase assay. Work is in progress to identify this kinase, as well as p45 and p48, and to determine their roles in agonist/antagonist-mediated ER function in hormone responsive breast cancer cells.

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INTRODUCTION

The regulation of gene expression in eukaryotic cells by steroid hormones involves the interaction of specific intracellular receptor proteins with the genome, resulting in the activation of tissue-specific gene networks. As a consequence, DNA synthesis is altered, as well as the synthesis of specific RNAs and proteins involved in cell proliferation, differentiation and physiologic function and development in diverse tissues and species. In addition, female steroid hormones and their receptors are involved in the regulation of abnormal growth in various tumors and tumor cell lines (1). For breast cancer, the elucidation of the molecular mechanisms responsible for the hormonal regulation of cell proliferation has been the object of intense research. Because most breast cancers are initially dependent upon estrogens for continued growth, much of this research has focused on the role of estrogen receptor (ER) in the control of gene expression and mitosis (2), and on its use as a marker for hormone responsiveness and prognosis (3). Equally important, and unknown, are the mechanisms by which estrogen responsive cancers escape regulation and progress to more aggressive and autonomous cancers. It is likely that multiple events are responsible, some of which may be sequentially linked and some of which may be independent. Mutations in the estrogen receptor gene, for example, have been implicated in altered regulation of gene expression(4, 5), although it is not yet known if such mutations are involved in tumor progression *in vivo*. Overexpression of various growth factors (eg. c-Myc) (6), or oncogenes (eg. HER2/neu) (7), both of which are regulated by ER, and/or loss or mutation of suppressor genes (eg. p53) (8) have also been implicated both in the initiation and progression of breast cancer. Again, the mechanisms by which gene amplification, overexpression, or silencing participate in these processes are not well understood. The rationale underlying this study is that information about the structure, composition, and cellular interactions of ER in normal and neoplastic cells will contribute to a better understanding of the molecular mechanisms that are responsible for hormonal control of cell proliferation.

The Steroid Receptor Superfamily

An improved understanding of the function and regulation of ligand dependent transcription factors is emerging from studies of the structure, composition and dynamics of the receptor proteins and the genes that encode them. The cloning and molecular analysis of the known steroid receptors has led to the definition of common functional domains by which they interact with responsive genes in hormone sensitive tissues (9-12). As a consequence of these interactions, DNA synthesis is altered, as well as the synthesis of specific RNAs and proteins involved in cell proliferation, differentiation and physiologic function and development. One member of this family of transcription factors is the estrogen receptor (ER), which mediates estrogenic responses

in diverse tissues including the brain, mammary gland, tissues of the reproductive tract, and cancers derived from some of these tissues (1).

Activation of receptors

All of the steroid receptors, including ER, are activated by one or more ligands and bind with high affinity and specificity to short *cis*-acting DNA sequences called hormone response elements (HREs). Interaction of steroid-receptor complexes with responsive genes *in vivo* can result in either induction or suppression of transcription, depending upon the target gene and the tissue (9, 13, 14). The molecular mechanisms by which either pathway occurs are still obscure, although it is generally accepted that for transcriptional activation, receptor-DNA complexes recruit, or facilitate the recruitment of, other transcription factors that comprise a functional transcription complex (11, 15). This process involves protein-protein interactions between receptor and other factors, which may be either general (eg. TFIIB) (16), tissue-specific (certain cofactors) (17), or receptor-specific (eg. for N-terminal domain of PR B isoform) (18). Some of these interactions may result in the formation of DNA loops (19) to accommodate long stretches of DNA between promoters and HREs, or possibly by altering the local chromatin organization (20, 21) to permit access of other transcription factors. DNA bending may also be involved (22, 23). It has also been suggested that nonhistone protein acceptor sites (24, 25) that are part of the nuclear matrix play a key role in receptor action, possibly by directing receptor to a target gene. Although such sites have been described, they have not yet been linked in an obligatory manner to a functional transcription complex *in vivo*. Obviously, all or any combination of these processes could occur.

Receptor-associated proteins

Although it is widely believed that an allosteric alteration of receptor structure occurs following hormone binding, exposing the DNA-binding domain, the nature of this change is still not understood. The participation of other proteins, both prior to and after hormonal activation, has been the subject of much investigation (26). At least three members of the heat shock protein family have been identified as putative accessory proteins by virtue of their association with several receptors *in vitro*. One of these, hsp90, has been implicated in the *in vitro* stabilization of the inactive form of receptors for glucocorticoids (GR) (27, 28), progestins (PR) (29) and estrogens (ER) (30). In support of the hypothesis of an essential role for hsp90, recent experiments in which expression of the hsp90 gene was conditionally regulated in *Saccharomyces cerevisiae* demonstrated that reduced levels of hsp90 severely compromised GR transcriptional activity (31). ER activity was less affected in this model. In contrast, members of the thyroid (32) and retinoid (33) receptor family do not appear to associate with hsp90, but rather are synthesized in an active

form that is able to bind to HREs *in vivo* in the absence of ligand. hsp56, which was recently identified as an immunophilin, also appears to be part of the unactivated complex of several steroid receptors (34) and may (35) or may not (36) be able to modify the transcriptional activity of some receptors (e.g. PR or GR) in response to immunosuppressants such as FK506. It has also been suggested that p59 (p56) may be the nuclear localization signal-binding protein (34). Another heat shock protein, hsp70, has been shown to bind to both PR and GR in the absence of hormone, but unlike hsp90, this association appears to be maintained, at least in part, in the activated receptor complex after hormone treatment (37). A recent study using baculovirus overexpressed human GR in *Spodoptera frugiperda* cells suggested that hsp70 is associated with the GR-GRE complex (38). However, Onate et al. (39) observed that hsp70 was not present or involved in specific recognition of a progesterone response element (PRE) by PR. Clearly, the role of hsp70 in ER, PR, and GR function remains unresolved.

An additional class of receptor-associated proteins are those that interact with the activated receptor complex. Thus, a 55-kDa nuclear accessory factor (NAF) has been reported to be essential for maximal binding of the vitamin-D receptor to the vitamin-D response element from the human osteocalcin promoter (40). Similarly, a 65-kDa factor termed triiodothyronine receptor-auxiliary protein (TRAP), which exhibits limited independent DNA binding, has been shown to enhance TR binding to DNA (41). More recently, both RXRα and RXRß have been shown to function as NAF- (42) and/or as TRAP-like proteins (43) by forming heterodimers with VDR, RAR, and TR. Such heteromers can have both positive and negative transcriptional activity (10). In addition, one or more members of a heteromeric complex may interact with mixed DNA elements or half sites in a responsive gene (44). In regard to other interactions with activated receptors, the non-histone high mobility group chromatin protein, HMG-1, has been shown to substitute for an unidentified factor present in partially purified progesterone receptor fractions that is responsible for promoting PR-DNA binding (45). Also, an unidentified single strand DNA binding protein has been implicated as being necessary for high affinity binding of ER to the vitellogenin A2 ERE (46). Recently, a protein called TIF1 (transcriptional intermediary factor) was isolated (47). TIF1, which stimulates RXR transcriptional activity, was also shown to interact with vitamin D, progesterone and estrogen receptors. Significantly, interaction with all receptors required a conserved region of the AF-2 domain of nuclear receptors and the interaction was ligand dependent. The authors propose that TIF1 serves as a coactivator to mediate the ligand-dependent AF-2 activity of nuclear receptors. Similarly, a yeast Sug1 homolog, designated Trip1, was recently implicated as a coactivator for thyroid hormone receptor (48).

One of the best studied systems is the interaction of receptors with members of the AP-1 family

of transcription factors (49). AP-1 is a protein complex composed of c-Jun and c-Fos whose activity is modulated by growth factors, cytokines, oncogenes and tumor promotering agents that activate protein kinase C. AP-1 induces transcription through interaction with a specific DNA recognition sequence, the 12-O-tetradecanonyl-phorbol-13 acetate (TPA) responsive element (TRE). This binding site is recognized by c-Jun and c-Fos heterodimers or c-Jun homodimers that are formed through a leucine zipper domain. Glucocorticoid receptor and AP-1 interactions have been observed at sites in the proliferin and collagenase promoters, as well as other TPA responsive genes. Subsequent analysis with other members of the family, including the thyroid hormone receptors (50, 51) , retinoic acid and vitamin D receptors (52), and estrogen receptor (49, 53), have also shown that AP-1 interactions can contribute to hormonal regulation. One of our goals is to better define the existence, identity, and role of ER-associated proteins in estrogen regulation of gene expression in hormone responsive tissues and cancers.

Estrogen agonists and antagonists

The nature of agonist- vs antagonist-receptor interaction and the resulting altered transcriptional activity are also still poorly understood at present. It is very likely that an altered conformation of receptor occurs in the presence of an antagonist (54), which could affect receptor stability (55), DNA binding (56)(57), interaction with other transcription factors such as AP1 (51, 53), phosphorylation (58), or interaction with hsp90 or hsp70. It has been proposed for ER that partial antagonists restrict TAF-2 activity, but not TAF-1 activity and that the promoter and cell context is therefore crucial for agonist vs antagonist activity (59-61). For both ER (62) and PR (63, 64), the pattern of phosphorylation appears to be essentially the same in the presence of either agonist or partial antagonists, suggesting that altered phosphorylation may not reflect agonist vs antagonist activity. For estrogen receptor, it has been proposed that higher order protein-protein interactions differ when DNA-bound ER is associated with estradiol vs 4-hydroxytamoxifen (65). It has also been suggested that some antagonists, like the "pure" estrogen antagonist ICI-164 (or the closely related ICI-182,780), may interfere with dimerization (66) or promote receptor degradation (55). It has recently been observed that the carboxyterminal tail of at least several steroid receptors may be a critical determinant of receptor response to ligand and that antagonism may result from a failure of antagonists to interact with a 30 amino acid domain at the carboxy terminus (67, 68). Obviously, there are still a number of key dynamic and molecular aspects of ligand-mediated receptor activity that are not resolved at this time.

AIMS

Based on our own data and the results of others, we wish to test the hypothesis that accessory

proteins are required for ER interaction with HREs and/or transcriptional initiation complexes and that estrogens and estrogen antagonists promote altered ER-accessory protein interactions that lead to differential transcriptional activity.

Specific Aims:

1. To identify and characterize ER-associated proteins isolated by affinity chromatography. We will use affinity chromatography methods to purify and characterize proteins that associate with hER expressed in CHO-ER cells and in MCF-7 breast cancer cells. In particular, we will use N-terminal and/or peptide microsequencing techniques to identify two proteins (45 & 48 kDa) that are selectively retained when hER is isolated by site-specific DNA adsorption to ERE-Agarose. The effect of each of these accessory proteins on hER-ERE interactions will be determined by gel retardation analysis with reconstituted complexes.

2. To use GST fusion proteins to identify and map interactions between ER and accessory proteins. Because important ER accessory or intermediary proteins may be tissue specific, limiting, or may bind less avidly to ER than those already isolated, we will use more sensitive in vitro methods to isolate additional ER associated proteins. Bacterially expressed glutathione-S-transferase-ER fusion protein (GST-ER) bound to glutathione-Agarose will be used to adsorb cytosol or nuclear proteins derived from T47D or HeLa cells. With this method, a large excess of uncomplexed hER can be used as a trap for proteins that form heteromeric complexes with ER. We will also use the same system to map the functional domains of ER that participate in specific interactions with identified accessory proteins. These studies will be carried out with GST-X fusion proteins (eg. X = p55 or hsp70) and a series of ^{35}S-labeled mouse and human ERs that contain specific point and deletion mutations.

3. To use a yeast transcription reconstitution system to identify, map, and characterize interactions between ER and accessory proteins. A yeast in situ "two-hybrid" system will be used both to identify novel estrogen receptor-associated proteins, from an MCF-7 cDNA library, and to characterize and map those already identified by the above in vitro approaches. This method allows ER-protein interactions to be characterized in vivo through the reconstitution of a Gal4 transcriptional activator.

4. To determine the molecular mechanisms by which accessory proteins mediate ER activity in vivo. hER activity will be analyzed in *s. cerevisiae* in the context of accessory protein gene knockouts, conditional expression mutants, or partial deletions of accessory protein

sequence to determine the role of ER-associated proteins (eg. hsp70 and p55) in ER transport and/or transcriptional activity.

In total, these studies should reveal new information about the molecular mechanisms of ER action and the role of accessory proteins in receptor-mediated transcriptional activation and suppression in hormone responsive tissues and cancers.

This annual report discusses progress with Aims 1 & 2 during the first year of funding.

MATERIALS AND METHODS

Culture of Mammalian Cells

CHO-ER cells (69) were cultured in Dulbecco's Modified Eagle Medium/Ham F-12 Nutrient Mixture (1:1) without phenol red with 10% iron-supplemented newborn calf serum that did not require charcoal treatment. 44 mM $NaHCO_3$, 1X antibiotic-antimycotic liquid (penicillin, streptomycin, and amphotericin), and 5 mg/L insulin were also added. To maintain expression and selection of the ER gene, 50 µM $ZnSO_4$ and 40 µM $CdSO_4$ were also included in the medium. CHO-k1 cells were cultured in the same medium without the addition of $ZnSO_4$ and $CdSO_4$.

MCF-7 cells were grown in Dulbecco's Modified Eagle Medium with phenol red supplemented with 10% calf serum that did not require charcoal treatment. 44 mM $NaHCO_3$, 1X antibiotic-antimycotic liquid (penicillin, streptomycin, and amphotericin), 5 mg/L insulin, 1X minimum essential media vitamins and 1X sodium pyruvate were also included in the medium. All cells were grown at 37•C in a humidified, 5% CO_2 atmosphere.

Whole Cell Ligand Binding Assay

The whole cell ligand binding assay was used to measure estrogen receptor levels within the cell prior to lysis. Cells were seeded into 6-well plates at approximately 2 – 3 x 10^5 cells per well, such that the cells would be subconfluent on the following day. After 12-18 hours, two wells were stimulated with 10 nM [6,7-^{3}H]-estradiol and two wells were competed with both 1 µM estradiol and10 nM [6,7-^{3}H]-estradiol in complete media. The cells in the remaining wells were left in complete media. All cells were incubated for 45 minutes at 37•C. After the incubation period, the media was removed by aspiration and each well was washed twice with 1 ml of phosphate buffered saline, pH 7.1, (PBS). Bound steroid was released from the cells by incubation with 1 ml of 100% ethanol for one minute. The ethanol was removed and collected in a

scintillation vial. A second ethanol elution was performed and added to the first. Scintillation fluor was added to the ethanol and the samples were counted on the tritium channel of a Tricarb scintillation counter. The counts present in the competed samples were subtracted as background from the uncompeted samples. The resulting value was used to determine the number of receptors present in the cells. The unstimulated cells in the remaining two wells were released with 0.2 ml of 1X trypsin. These cells were pelleted by centrifugation at 800 rpm, resuspended in 0.1 ml of PBS and live cells were counted in the presence of trypan blue. The cell count was used in conjunction with the previous calculation of receptor number to determine the number of receptors present per cell.

Cell Fractionation

For the preparation of cytosolic and nuclear extracts, subconfluent cells were released from tissue culture vessels with a non-enzymatic cell dissociation solution. The releasing action was inactivated by the addition of serum containing media. The cell suspension was collected in tubes and the tissue culture vessels were rinsed with PBS to obtain any remaining cells. Cells were pelleted gently at 800 rpm for 5 minutes, washed three time with PBS and pelleted as above to remove any remaining cell dissociation solution. The cells were then resuspended ten volumes of PBS that contained 10% glycerol and 60 nM [6,7-^{3}H]-estradiol and incubated for 30 minutes at room temperature with rocking. The cells were then pelleted as before and the incubation repeated with a fresh aliquot of the PBS-[6,7-^{3}H]-estradiol solution. The packed cell volume of the cell pellet was noted following this final incubation. The pellet was resuspended in four times the packed cell volume of 50 mM Tris, pH 7.8, 2 mM DTT (salt-free buffer) also containing a protease inhibitor cocktail (leupeptin, chymostatin, pepstatin A, antipain, aprotinin, PEFABLOC). The cells were homogenized in an ice bath by dounce homogenization (type B pestle) with 5 strokes every 5 minutes for 20 minutes. The homogenate was centrifuged for 30 minutes at 10,000 rpm at 4•C. The supernatant was collected as cytosol 1. The pellet was resuspended in a fresh aliquot of salt-free buffer to wash the pellet and remove any remaining cytosolic proteins. The homogenate was centrifuged as described for cytosol 1. The supernatant was collected as cytosol 2. The crude nuclear pellet was then resuspended in four times the original packed cell volume in 50 mM Tris, pH 7.9, 2 mM DTT, 400 mM NaCl (high salt buffer) containing the protease inhibitor cocktail described above. The nuclear pellet was allowed to extract for 60 minutes on ice with 5 strokes every 15 minutes. The homogenate was centrifuged as for the cytosolic fractions. The supernatant was collected as nuclear fraction 1. The extraction was repeated with two times the packed cell volume of high salt buffer and centrifuged as before. The supernatant was collected as nuclear fraction 2. The receptor content in the cytosolic and nuclear fractions was measured by direct counts and by adsorption to controlled pore glass beads (described below). The nuclear fractions were clarified by centrifugtion for 30 minutes at 50,000 x g in an ultracentrifuge and stored at -80•

C.

For whole cell extractions, crude cell pellets were extracted with high salt buffer only, as described above for the nuclear pellet.

Controlled Pore Glass Bead Assay For Steroid Binding

An assay using controlled pore glass beads for the quantitation of receptor-bound steroid was developed previously in the lab (70). Controlled pore glass bead columns were prepared by cutting Whatman glass microfibre paper GF/A with a #4 cork punch to fit tightly in disposable syringes. The luer tip of the disposable 2.5-ml glass hypodermic syringes were fitted with a 16 gauge stainless steel needle approximately 2.5-cm long with the sharp tip cut off. The needle-fit syringes were placed held vertically by a rack. A single filter paper was pushed to the bottom of the syringe and moistened with a few drops of 10 mM Tris pH 7.4, 1 mM NaN_3 ($T_{10}N_1$). 400 µl of controlled pore glass (CPG) bead suspension (equal volume of beads and $T_{10}N_1$) was pipetted onto the filter paper. A second filter paper was placed on top of the beads. The columns were then placed in the cold room at 4•C and washed twice with 1-ml aliquots of 10 mM Tris, pH 7.4, 0.4 M KCl ($T_{10}K_{400}$). The wash solution was placed on the column at the top of the syringe to wash down any beads clinging to the sides. A measured aliquot of a sample labeled with excess [6,7-^{3}H]-estradiol was placed onto the column and allowed to drain into the column. Two 1-ml washes $T_{10}K_{400}$ were placed on the column. Again, the wash solution was applied from the top so that the column sides were cleaned. The wash solution was allowed to drain completely into the column before the next wash was added. One 20-ml wash of $T_{10}K_{400}$ was applied following the two above washes. A plastic disposable 20-ml syringe fitted with tygon tubing was used as a reservoir. After the washes were complete, the columns were brought out of the cold and allowed to warm to room temperature for 20 minutes. The wash reservoirs and needle tips were removed while warming. 20 ml disposable glass vials were placed under each column to collect the eluate. To elute the bound [6,7-^{3}H]-estradiol, two 1-ml aliquots of absolute ethanol were applied sequentially to the columns. 10 ml of scintillation fluid was added to each pooled eluate and the samples were counted on the tritium channel in a Tricarb scintillation counter. An empirically determined quench factor of 0.805 was used in all calculations to correct for the presence of ethanol in the samples. In addition, an efficiency factor of 2.13 for tritium was also empirically determined.

SDS-Polyacrylamide Gel Electrophoresis and Staining

Protein samples were analyzed by separation on reducing 10-12% SDS-polyacrylamide gels (12.5 x 14 x 1.5 cm) that contained 0.39% N,N'-diallyltartardiamide, 0.375 M Tris, pH 8.8, 0.1% SDS, 0.035% ammonium persulfate, and 0.025% N,N,N',N'-tetramethylethylenediamine in the separating gel. Before electrophoresis, samples were diluted in Laemlli sample buffer (50 mM Tris, pH 6.8, 10% sucrose, 2% SDS, 5% ß-mercaptoethanol, and 0.005% bromophenol blue) and heated at 95 C for 5 minutes. Aliquots of each sample were applied to the wells of a 7% polyacrylamide stacking gel and electrophoresis was carried out at 4 C overnight at 10 mA in 25 mM Tris, 250 mM glycine and 0.1% SDS.

Immunoblotting

For Western blot analyses, separated proteins were transferred electrophoretically from the gel to nitrocellulose by a modified method of Towbin (71). Electrophoretic transfer was carried out at 4• C for 2 hours at 1.0 A in a buffer that contained 0.025 M glycine, 0.192 M Tris and 10% methanol. Following transfer, the nitrocellulose was stained with Ponceau S to visualize transferred proteins and lanes that contained molecular weight markers were cut out. The nitrocellulose blots were treated with a filtered solution of 3% Carnation nonfat dry milk dissolved in Tris-buffered saline, pH 7.4 containing 0.02% Tween 20 (TBS-Tween) for 1 hour to saturate the nitrocellulose with protein. A second incubation was carried out with a monoclonal antibody for ER (4 µg/mL) (70), and hsp70 (1:2000) (72), or with a rabbit or mouse (73) polyclonal antibody for p55 (1:2000) in 1% milk in TBS-Tween for 1 hour at 22 C. Incubation of the blots for an additional hour was performed with 2 µg/ml of either rabbit anti-rat IgG (ER), rabbit anti-mouse IgG (hsp70, p55) or biotinylated goat anti-rabbit IgG (p55) prior to a final 1 hour incubation in a 1:1000 dilution of either HRP-Protein A (horse radish peroxidase) or ^{125}I-Protein A (ER, hsp70) or HRP-Streptavidin (p55). After each incubation, blots were washed with TBS-Tween. For blots that were incubated with HRP-Protein A, immunoreactive proteins were visualized by incubation with 5 mg of diaminobenzidine in 10 ml Tris-buffered saline, pH 7.4 in the presence of 30 µl 30% H_2O_2. Proteins on blots incubated with ^{125}I-Protein A were visualized by autoradiography.

Polyacrylamide Gel Silver Stain Analysis

For silver stain analysis of proteins, polyacrylamide gels were fixed in isopropanol-acetic acid-water (2:1:8) for 1 hour followed by 30 minutes of continuous washing with running distilled water. Gels were then fixed in 10% glutaraldehyde for 15 minutes. Following the glutaraldehyde fixation the gels were again washed with distilled water for 3-5 hours. After washing, gels were

stained with silver for 15 minutes in a solution containing 0.07% NaOH, 1.38% NH_4OH, 14.8% ethanol, and 0.8% $AgNO_3$. Gels were then washed under running distilled water for 20 minutes. The proteins were visualized by development of gels in a solution of 10% ethanol, 0.005% citric acid, and 0.0185% formaldehyde until staining was of the desired intensity. Following development, the gels were washed with repeated changes of glass distilled water. After 2 hours, gels were placed in fresh glass distilled water and allowed to wash overnight. Once washing was complete, gels were dried between two sheets of porous cellophane by forced warm air.

Construction Of Affinity Chromatography Columns

Three different affinity chromatography columns were constructed for the purification of estrogen receptor from extracts of mammalian cells.

Preparation of an estradiol -Sepharose affinity column

The estradiol-Sepharose column was prepared previously in the lab as reported (70), except that a diglycidyl ether bridging moiety was used in place of the thiopropyl bridge described in the original procedure.

Preparation of an immunoadsorbent using CNBr-activated sepharose

An immunoadsorbent was prepared by coupling the desired antibody to CNBr-activated Sepharose (74).The required amount of freeze dried CNBr-activated sepharose was weighed (1 g gives about 3.5 mL swollen gel) and suspended in 10^{-3} M HCl. Once swollen, the gel was washed for 15 minutes 10^{-3} M HCl on a sintered glass filter (wash with approximately 200 ml/g of powder). The antibody was dissolved in 0.1 M $NaHCO_3$, 0.5 M NaCl (coupling buffer) using 5 mL per gram of powder swollen. The antibody was then mixed with the swollen gel in a stoppered vessel for 2 hours at room temperature. Following coupling, the excess antibody was removed by washing with coupling buffer. Active groups that remained on the column were blocked by incubation with 0.1 M Tris, pH 8.0 for 1 hour. Following blocking, the gel was washed with 3 cycles of alternating pH using 0.1 M acetate, pH 4.0, 0.5 M NaCl and 0.1 M Tris, pH 8.0, 0.5 M NaCl The adsorbent was stored at 4•C.

Coupling of a biotinylated ERE to avidin-agarose

A 27-bp oligonucleotide containing the estrogen response element (ERE) from the vitellogenin

A2 gene (GATCCTAGAGGTCACAGTGACCTACGA) (75) was synthesized with a 6 carbon spacer and a terminal amino group (Genetic Designs; Houston, TX). 10 nmol of the oligonucleotide was added to 0.1 M $NaHCO_3$, pH 9.1 in a final volume of 50 µl. 2.0 mg of amino-hexanoyl-biotin-N-hydroxysuccinimide ester was dissolved in 25 µl DMF and added, with vortexing, to the oligonucleotide solution and incubated for 90 minutes at 40• C. 125 µl water, 20 µl 3 M sodium acetate and 800 µl of absolute ethanol were added to the mixture. Following a 30 minute incubation at -70• C, the oligonucleotide was pelleted by centrifugation at 10,000 rpm for 15 minutes. The pellet was washed with 70% ethanol and dried in a speed-vac. The dried pellet was resuspended in 50 µl of water and mixed with an equal molar quantity of the complementary strand. TE buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA.) containing 0.2 M NaCl was added to yield a final volume of 100 µl. The mixture was heated to 95• C for 5 minutes in a heating block and allowed to cool slowly to room temperature (4 hours). Duplex DNA was precipitated and dried as described above. For later experiments a 37 base pair oligonucleotide containing the ERE (AGCTTGTCCAAAGTCA**GGTCACAGTGACC**TGATCAAA) described above was prepared commercially (Operon Technologies, Inc.) to contain the biotin modification with a 6 carbon spacer.

The avidin-agarose was equilibrated in 20 mM Tris, pH 7.9; 1 mM DTT, 100 mM NaCl. The amount of biotinylated ERE (B-ERE) needed was prepared as a 1 ml solution in the equilibration buffer just described. The OD_{260} of this solution was read to quantitate the amount of DNA prior to adsorption to the avidin-agarose. The B-ERE solution was applied to the avidin-agarose and incubated at room temperature with rocking for 15 minutes. The avidin-agarose was pelleted and the supernatant was removed. The OD_{260} was read as before. The decrease in the absorbance reflected the amount of DNA that had adsorbed to the avidin-agarose. The supernatant was reapplied to the avidin-agarose and incubated for an additional 15 minutes as before. Again, the avidin-agarose was pelleted and the supernatant collected. The OD_{260} of the supernatant was read as before. The procedure was repeated until the OD_{260} no longer decreased. The beads were then washed thoroughly with equilibration buffer to remove any uncoupled B-ERE remaining. The B-ERE coupled avidin-agarose was stored as a 1:1 suspension in equilibration buffer at 4• C.

Affinity Chromatography

Estradiol-Sepharose Chromatography

2.5 ml of CHO-ER whole cell extract, adjusted to contain 0.7 M NaCl and 1 M urea, was applied to a 200 µl estradiol-Sepharose column and incubated batchwise for 1 hour at 4• C. The

column was washed with 20 bed volumes each of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 700 mM NaCl, 1 M urea), and the same buffer with 400 mM NaCl and 3 M urea. Bound ER was eluted with 2 x 10^{-5} M [6,7-^{3}H]estradiol in a buffer that contained 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 200 mM NaCl, and 5 M urea. The yield of ER was determined by specific adsorption to CPG beads.

H222-Immunoaffinity Chromatography

2.5 mL of CHO-ER whole cell extract was adjusted to contain 1 M urea and labeled with excess [6,7-^{3}H]estradiol for 1 hour at 4• C. The extract was applied to 200 µl of H222-Sepharose beads in a 10 ml BioRad Econo Column and incubated batchwise for 1 hour at 4• C. The beads were then washed with 20 bed volumes of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 400 mM NaCl, 1 M urea). Bound ER was eluted in a buffer containing 50 mM Tris, pH 7.4, 2 M NaSCN, 10% DMF at 22• C. The yield of ER was determined both by CPG assay as well as by direct counting in scintillation cocktail.

DNA-affinity Chromatography

2.5 ml of CHO-ER whole cell extract was labeled with excess [6,7-^{3}H]estradiol for 1 hour at 4• C and then dialyzed against a buffer containing 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 M urea. ER content in the extract was determined by CPG assay. Excess biotinylated ERE (B-ERE) was added to the extract at a ratio of 5 pmol of ERE to 1 pmol of ER along with 50 µg poly(dIdC) and 10 µg of the progesterone response element from the mouse mammary tumor virus long terminal repeats (TGACTTGGTTTGGTACAAAATGTTCTGATCTG) as carrier DNA. This mixture was incubated for 20 minutes at 22• C, followed by an additional incubation for 40 minutes at 4• C, and applied to a 200 µl NeutrAvidin-agarose column and incubated batchwise for 1 hour at 4• C. The column was washed with 20 bed volumes of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 M urea). Bound ER was eluted in a buffer containing 20 mM Tris, 1 mM EDTA, 1 mM DTT, 1 M NaCl, 1 M urea and quantitated by CPG assay as well as by direct counting in scintillation cocktail.

For the isolation of the 48- and 45-kDa proteins, unlabeled CHO-ER whole cell extract was eluted from the B-ERE/Streptavidin-agarose beads in a buffer containing 700 mM NaCl and applied to the estradiol-Sepharose column. Subsequent chromatography on the estradiol-Sepharose column proceeded as described above.

When CHO-ER nuclear extracts were used, the procedure was the same as described except that incubation with [6,7-^{3}H]estradiol was not necessary because the cells were pre-labeled with

[6,7-^{3}H]estradiol in culture.

In Situ Chemical Cleavage Of Proteins With BNPS-Skatole

For microsequencing of the 55-kDa estrogen receptor associated protein, chemical cleavage of the purified protein was performed to obtain amino acid sequence analysis of internal peptide fragments. Proteins present in the hER complex eluted from the E-Seph column were separated by SDS-PAGE and transferred to Immobilon-P as described above. Following staining with Ponceau S, the 55-kDa band was excised, cut into small pieces and placed in a microfuge tube. BNPS-Skatole (1μg/μL) in 75% acetic acid was added and the tube was incubated at 47• C for 1 hour (76). The liquid was removed and the membrane was washed thoroughly with water to remove the acetic acid. The washed membrane was dried by vacuum centrifugation and incubated in a buffer containing 50 mM Tris, pH 9.1, 2% SDS, 1% Triton-X 100 for 2 hours at 22• C to elute the fragments. An appropriate volume of a 6X solution (60% sucrose, 100 mM DTT and 0.3% bromophenol blue) of diluent was added to the mixture and the incubation was continued for an additional hour. The eluted fragments were then separated by SDS-PAGE and transferred to Immobilon-P. Transferred fragments were visualized by staining with Coomassie Brilliant Blue R-250 and selected fragments were excised and sent to the University of Kentucky Macromolecular Structure Analysis Facility for sequencing. The results of the sequence analysis of the peptides were as follows: CHO-ER fragment 1 - AAPEEEDNVLVLKKSNFKEALAA; CHO-ER fragment 2 - NYLLVEFYA; CHO-ER fragment 3 - LAKQTGPAATTL; MCF-7 fragment 1 - APEEEDHVLVLRKSNFA.

Preparation Of Peptide Antibodies In Rabbits

A peptide antibody was prepared to the first 22 amino acids (CHO-ER fragment 1) of the 55 kDa protein described above to facilitate further analysis of this estrogen receptor-associated protein. 2 mg of peptide and 5 mg ovalbumin were dissolved in 1 ml PBS. To this mixtur, 1 ml of 21 mM glutaraldehyde was added dropwise with stirring and allowed to stir overnight at room temperature. The coupled peptide was aliquoted into 400 μl fractions and stored at -20•C for injections. For the first injection, the peptide-conjugate was emulsified with an equal volume of Freund's complete adjuvant and injected at the right and left flanks into a New Zealand White rabbit using an 18 gauge needle. Subsequent boost were peformed every two weeks with the peptide-conjugate emulsified in Freund's incomplete adjuvant.

For bleeding the rabbits, the central ear artery was distended with methyl salicylate. The collected blood was allowed to clot at room temperature for 10 mintues and then condense on ice

for another 10 minutes. The blood was centrifuged at 10,000 rpm for 20 minutes. The supernatant represented the serum and was collected. The immunoglobulin fraction was precipitated with 40% ammonium sulfate by the addition of saturated ammonium sulfate dropwise to the stirring the serum. After 30 minutes the precipitate was pelleted by centrifugation at 10,000 rpm for 30 minutes. The supernatant was discarded and the pellet was dissolved in PBS.

For purification of the peptide antibody, a peptide adsorbent was prepared from 2 ml of swollen CNBr-activated Sepharose, as described earlier for the preparation of the immunoadsorbent. 4 mg of peptide was dissolved in 0.1 M $NaHCO_3$, 0.5 M NaCl. The peptide was incubated batchwise with the Sepharose for 2 hours at 22• C. The column was washed with 25 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl and allowed to drain to top of column bed. 3 ml of 0.1 M Tris, pH 8.0 was added to the column and incubated batchwise for 2 hours to block remaining active groups. The column was drained and then washed 3 times with alternating pH using 0.1 M acetate, pH 4.0, 0.5 M Nacl and 0.1 M Tris, pH 8.0, 0.5 M NaCl. The column was stored at 4• C in 0.1 M Tris, pH 8.0, 0.5 M NaCl.

The ammonium sulfate precipitated immunoglobulin fraction was applied to the peptide adsorbant described and incubated batchwise for 2 hours at room temperature. The column was drained and washed with PBS until the OD_{280} reading of the flow through was less than 0.2. The antibody was eluted with 0.2 M glycine, pH 2.8 in 1 ml increments, with the fractions collected into tubes containing 75 µl of 1 M Tris. The OD 280 was determined for each fraction to locate the eluted antibody. The OD_{280} divided by 1.4 represents the yield of eluted antibody in milligrams ($e^{280} = 1.4$). The column was washed to neutral pH with PBS and was used for multiple rounds of antibody purification.

Metabolic Labeling Of CHO-ER Cells With ^{35}S-Methionine

CHO-ER cells were seeded into 6 well plates in complete medium and incubated as described earlier. 12-18 hours later the medium was removed and the cells were washed 2 x 2 ml with PBS. The cells were then incubated in methionine-free medium for 1 hour at 37• C in a humidified, 5% CO_2 atmosphere. This medium was removed and replaced with ^{35}S-methionine-containing medium (0.1 mCi/well) in the presence of either ethanol vehicle, 10 nM estradiol, 100 nM ICI 182,780 or 100 nM 4-hydroxytamoxifen and incubated for 2 hours. Cells were released with trypsin, washed thoroughly with PBS and lysed by freeze-thaw in a buffer containing 10 mM Tris, 1 mM EDTA, 1 mM DTT, 400 mM NaCl, 1 M urea and protease inhibitors (aprotinin, leupeptin and PEFABLOC). The mixture was passed through a 25 gauge needle to shear the DNA and

clarified by centrifugation at 10,000 rpm at 4• C for 20 minutes. The supernatant was collected and the protein concentration was determined by a modified Coomassie brilliant blue assay (77). Samples were adjusted for total protein concentration and hER complexes were subsequently isolated by either H222-Seph or B-ERE chromatography.

Labeling Of Oligonucleotides

Preparation of a radiolabeled oligonucleotide was performed for use in electrophoretic mobility shift and nitrocellulose filter binding assays. A 20 µl reaction mixture for labeling 10 pmol of oligonucleotide was assembled in a solution containing 50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 10 mM DTT, 1 mM spermidine, and 200 µCi of crude (γ^{32}P)-ATP. To this mixture, 5-8 units of T4 polynucleotide kinase was added and the reaction was incubated for 30 minutes at 37•C. Following this incubation, an additional 5-8 units of kinase was added and a second 30 minute incubation was performed. 1µl of the reaction mixture was removed and diluted ten-fold to use for the specific activity determination (see below). 2 µl of a 20% glycerol solution containing bromophenol blue was added to the remaining sample. The sample was then applied to a 9% polyacrylamide-bis gel containing 0.045 M Tris-borate, 0.001 M EDTA, pH 8.0 (0.5X TBE). The gel was run in 0.5X TBE for 1 hour at 10 mA. Following electrophoresis, one glass plate was removed and he gel was covered with plastic wrap. The gel was exposed to x-ray film for 10-20 seconds. The film was developed and used as a template to cut out the band corresponding to the desired radiolabeled double-stranded oligonucleotide. The excised gel slice was placed in a sterile eppendorf tube with a buffer containing 0.5 M NaAc and 1.0 mM EDTA and crushed with a polished glass rod. The sample was incubated at 37•C for 3 hours. The crushed acrylamide was separated from the supernatant by centrifugation through a disposable centrifugal microfilter. 10 µg of tRNA was added to serve as a carrier and 1 ml of absolute ethanol was added. The sample was place at -20• C for 2 hours and pelleted by centrifugation. The resulting pellet was washed with 70% ethanol, dried by vacuum centrifugation and resuspended in 10 mM Tris, pH 8.0, 1.0 mM EDTA.

To determine the specific activity of the labeled oligonucleotide, 1 µl of the 1:10 diluted sample was spotted onto a strip of polyethyleneimine impregnated cellulose. The cellulose was placed in a TLC chamber containing 0.5 M KH_2PO_4, pH 3.4. The oligonucleotide remained at the origin wherease ATP and inorganic phosphate migrate in the same direction as the solvent. The specific activity of the probe was determined by measuring the amount of radioactivity remaining at the origin.

Electrophoretic Mobility Shift Assay

The electrophorectic mobility shift assay (gel shift) was employed to visualize and examine protein-DNA complexes formed following incubation of partially purified estrogen receptor complexes with an estrogen response element. 10-25 fmol of ER (determined by specific adsorption to controlled pore glass beads) were incubated with 40-100 fmol of labeled oligonucleotide for 30 minutes at 22• C. All incubations were performed in the presence of 1 µg poly(dIdC), 25 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 20% glycerol. The total protein concentration in all incubations was normalized to 50 µg by the addition of BSA. The samples were analyzed on a non-denaturing, 5% polyacrylamide gel (0.5X TBE) for 2 hours at 10 mA. Radioactive bands were visualized on dried gels by autoradiography at -75 C with chromex intensifying screens for 3-12 hours.

Circular Permutation

The circular permutation vector B3ConsERE was digested with EcoRI, HindIII, EcoRV, NheI, BamHI to produce 427 base pair DNA fragments (23). Labeling and purification of the ERE-containing DNA fragments were carried out by members of the Nardulli lab, as previously described (23).

For electrophoretic mobility shift assays, 250 fmol of BERE and 100 fmol of ESeph and EATP partially purified ER complexes were incubated with 10 µg of BSA, 1µg of poly(dIdC) in a buffer containing 15 mM Tris, pH 7.9, 0.2 mM EDTA, 4 mM DTT, 80 mM KCl, and 10% glycerol for 15 minutes at 4• C. Labeled DNA fragments (10,000 cpm) were then added and the reaction was incubated for an additional 20 minutes at room temperature. Protein-DNA complexes were separated on low ionic strenth, 8% polyacrylamide-bis (75:1) gels at 300 V for 4 hours at 4•C with buffer recirculation. For supershift experiments, 250 ng of the monoclonal antibody H222 was included in the incubation mixture. Competition assays contained 45 ng of a 54 basepair DNA fragment containing either an ERE or a nonspecific DNA sequence. Quantitation of the migration of the ER-DNA complexes and free probe present in individual lanes was carried out using a Molecular Dynamics phosphoimager.

Nitrocellulose filter binding assay

In a 96-well microtiter plate, 30 µl reactions were prepared to contain 1 µg Poly(dIdC) and 30 µg bovine serum albumin in a buffer containing 20 mM Tris, pH 7.9, 1 mM DTT and 0.1 M NaCl (final concentration). 30 fmols of ER were delivered from either a crude extract or a partially purified fraction and incubated on ice for 5 minutes. Specific competitor, when used, was added at

this stage at the desired molar excess. The plate was then spun briefly in a refrigerated table top centrifuge at 800 rpm to bring all liquid to the bottom of the well. While on ice, 120 fmol of labeled oligonucleotide was added to the side of each well. The plate was again spun briefly to mix the probe with the reaction mixture. The reaction was then incubated for 30 minutes at room temperature. A 96 well nitrocellulose multiscreen plate was prepared in a vacuum manifold by prewetting each well with 20 mM Tris, pH 7.9, 1 mM DTT and 0.1 M NaCl (washing buffer). 100 µl of washing buffer was left in each well for sample dispersion. Following the 30 minute incubation samples were transferred from the microtiter plate using a multichannel pipetman into the appropriate well of the multiscreen plate. Once all samples were transferred, vacuum was applied to the manifold. All wells were then washed three times with 200 µl of washing buffer containing 0.01% NP-40. Once washing was complete, the vacuum was increased to dry the membranes. When dry, the multiscreen plate was removed from the manifold and 40 µl of scintillation fluor was added to each well. The plates were counted in a Packard Top Count microtiter plate scintillation counter.

Production of GST-hER Fusion Proteins

GST-hER expression vectors were transformed into the BL21-plys strain of E.coli. Overnight cultures were diluted 1:10 and grown at room temperature in selective media (LB, 50 µg/ml ampicillin). Cells were induced with isopropyl-ß-D-thiogalactoside (0.1 mM final concentration) at an absorbance of 1.2 (at 600 nm). After two hours, bacteria were collected by centrifugation, resuspended in four volumes of lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM DTT) containing protease inhibitors (leupeptin, chymostatin, pepstatin A, antipain, aprotinin and PEFABLOC), sonicated, and centrifuged. Receptor levels were determined by controlled-pore glass bead (CPG) assay.

Metabolic Labeling of T47D Cells with ^{35}S-Methionine

T47D cells were grown to approximately 80% confluency and were metabolically labeled for two hours in media containing [^{35}S]methionine (100 µCi/ml; ^{35}S-Pro-Mix, Amersham). Cells were released, pelleted, and washed twice in PBS. Whole cell extracts were prepared by adding four volumes of lysis buffer (20 mM Tris-HCl at pH 7.4, 400 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.1% NP-40, and protease inhibitors). The cell suspension was incubated for 10 minutes to complete lysis and centrifuged at 10,000 rpm for 20 minutes to clarify. The supernatant was collected and the protein concentration determined by a modified Coomassie blue assay.

GST-hER Pulldown Assay

^{35}S-labeled mammalian whole cell extracts were precleared overnight at 4°C by incubation with 100 µl of glutathione-agarose beads (Sigma) preloaded with 200 µl GST extract alone. 50 µl of beads were preloaded with 150 µl of either GST alone or GST-LBD extracts for 2 hours at 4°C. Samples were washed five times with 0.1 M sodium phosphate/0.4 M NaCl/3.0 M urea. Equal volumes of the precleared mammalian extracts were then incubated with the beads for 3 hours at 4°C in the absence and presence of 1 µM estradiol (E2), 4-hydroxy-tamoxifen (OHT), or ICI 182,380 (ICI). After five washes in phosphate buffered saline/0.5% NP-40, the proteins were eluted with 50 µl Laemlli sample buffer and resolved on a 12% SDS/polyacrylamide gel. Bands were visualized in dried gels by autoradiography.

In Vitro Kinase Assay

50 µl of glutathione-agarose beads were preloaded with 150 µl of either GST alone or GST-LBD extracts for 2 hours at 4°C in the absence and presence of 1 µM estradiol (E2), 4-hydroxy-tamoxifen (OHT), or ICI 182,380 (ICI). Samples were washed five times with 0.1 M sodium phosphate/0.4 M NaCl/3.0 M urea. Whole cell extracts of T47D cells were diluted so that the final composition of the buffer was 20 mM Tris-HCl at pH 7.4, 75 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 20 mM ß-glycerolphosphate, and 0.1 mM Na_3VO_4. Diluted extracts were mixed with the preloaded beads, incubated for 3 hours at 4°C, and washed with washing buffer (20 mM HEPES at pH 7.7, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA and 0.05% NP-40). The pelleted beads were resuspended in 150 µl kinase buffer (20 mM HEPES at pH 7.6, 20 mM $MgCl_2$, 20 mM ß-glycerolphosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM Na_3VO_4 and 2 mM DTT) containing 20 µM ATP and 25 µCi of [γ^{32}P]ATP. After 45 minutes at 30°C, the reaction was terminated by washing five times in HEPES binding buffer (20 mM HEPES at pH 7.7, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% NP-40, 0.5 mM DTT, 20 mM b-glycerolphosphate, and 0.1 mM Na_3VO4). Proteins were eluted with 50 ul of Laemlli sample buffer and resolved on a 12% SDS-polyacrylamide gel, followed by autoradiography.

RESULTS AND DISCUSSION

As discussed earlier, a wealth of data implicates the interaction of ER with other cytoplasmic and nuclear proteins, both before and after activation by hormone and/or specific binding to DNA enhancer elements in responsive genes. An objective that has evolved from our studies on the

DNA-binding properties of hER, as well as from the overexpression and purification of hER from mammalian cells, is to determine the role of receptor-associated proteins in DNA binding, transcriptional activity and/or cytoplasmic-nuclear transport of ER. In addition to the association of several heat shock proteins with unactivated receptors, described above, other proteins have been implicated in the specific recognition of DNA response elements on target genes by receptors (46) and as mediators of transcriptional activity (16, 78). It is also clear that the TAF-1 and TAF-2 functions of ER are not identical and that their respective roles in transcriptional activation/repression are therefore almost certainly mediated by different intermediary factors. These factors may be cell- and promoter-specific as well as limiting, as suggested for PR TAF-1 (78), or general, as suggested for TAF-2 (TFIIB)(16). However, it is still unclear how many different factors participate or how steroid receptors influence the formation or stability of transcription initiation and/or elongation complexes.

Isolation and Identification of hER-Associated Proteins

To isolate, identify, and characterize both cytoplasmic and nuclear ER-associated accessory proteins, we have used immunoaffinity (H222-Seph), steroid affinity (E-Seph), and site-specific DNA (B-ERE) affinity chromatography to identify proteins that co-purify with hER in extracts from MCF-7 human breast cancer cells and CHO cells stably transfected with hER (CHO-ER cells) (**Fig. 1**). Analysis of eluted proteins by SDS-PAGE revealed similar, but not identical, patterns of associated proteins for each of these techniques (**Table 1**). In each case, the expected 66-kDa hER was observed in silver stained gels, as well as on a western blot. In addition to hER, a 70-kDa band was retained by all three methods. Western blot analysis identified this species as hsp70. Treatment of CHO-ER extract *in vitro* with ATP reduced the amount of ER-bound hsp70, similar to the result reported by Smith and Toft for chick PR treated with ATP (26, 79).

An additional species that co-purified with hER by all three methods was a 55 kDa protein (**Table 1**) that we have recently identified by N-terminal amino acid sequencing as protein disulfide isomerase (PDI) (80), or thyroid hormone binding protein (p55) (81). It is not yet clear what role PDI may have in ER action. However, this ubiquitous and abundant protein is essential for yeast viability (82) and a recent report suggests that one important function of PDI is to catalyze disulfide bond formation and rearrangements within kinetically trapped, structured folding intermediates (83). Interestingly, deletion of the catalytic domain of PDI is not lethal in yeast, (84), suggesting that the essential role of PDI lies in a different function. One possibility is that PDI may serve as a chaperone in the cytoplasmic/nuclear transport of proteins, since a subpopulation of p55 has been localized to the nuclear membrane of human A431 and rat GH_3

cells (73).

Two additional protein bands, which migrate at 45 and 48 kDa (p45 and p48), were observed in eluates containing hER purified on B-ERE-agarose (**Table 1**). Although these two bands are also present to a lesser extent in immunoaffinity eluates, their intensity is significantly enhanced when ER is bound to ERE, suggesting that their association with hER is promoted or stabilized by ER/ERE interaction. One of these species may be a 45-kDa single strand DNA-binding protein identified earlier by Mukherjee (46), as mentioned above. A virtually identical pattern of ER-associated proteins was observed by SDS-PAGE with affinity-purified whole cell extracts from MCF-7 breast cancer cells, indicating that the four co-purified proteins obtained from CHO-ER cell extracts are not specific to CHO-ER cells, and that the presence of an artifactual variant of hER (G400V)(85) in these CHO-ER cells is not a limitation. Because the CHO-ER cells express very high levels of hER (2-3 x 10^6 molecules/cell) (69), it is a good model for studies of ER-associated proteins.

Effects of Estrogen Agonists and Antagonists

To examine possible differential effects of estrogen agonists and antagonists *in situ* on the interaction of hER with the associated proteins listed in **Table 1**, cells were metabolically labeled with ^{35}S-methionine prior to treatment with estradiol (E2), ICI-182,780 (ICI-182), or 4-hydroxytamoxifen (OH-Tam). hER was then isolated on B-ERE-Agarose or H222-Sepharose. None of the tested ligands (E2, ICI-182, OH-Tam) had any effect on the stoichiometry of protein association with hER purified by adsorption to B-ERE. The same 45-, 48-, 55-, and 66-kDa (hER) bands that were observed by silver stain were seen in the autoradiogram except for ^{35}S-hsp70, which was absent due to the low turnover rate of hsp70. However, on western blots, hsp70 was readily observed, as were hER (66 kDa) and PDI (p55). Like the other three associated proteins (**Table 1**), the hsp70/hER stoichiometry was constant for each *in situ* treatment.

In contrast to the B-ERE chromatography results, when total hER complexes were isolated by immunoadsorption (H222-Sepharose), a significant reduction in the amount of associated hsp70 was observed following treatment of CHO-ER cells *in situ* with either estradiol or the partial antagonist OH-tam (**Table 1**), whereas dissociation of hsp70 did not occur in the absence of ligand or when cells were treated with ICI-182, a complete estrogen antagonist. In contrast to other published reports on the effect of ICI-164 (an analog of ICI-182) on ER stability in mouse uterus (55), no significant loss of ER was observed in extracts of CHO-ER cells treated with ICI-182. The hsp70/ICI-182 results suggest 1) that hsp70 may be required for high affinity

ER/ERE binding and 2) that a subpopulation of ER that is competent for DNA binding remains associated with hsp70. It has been reported that treatment of chick PR with hormone *in vitro* partially disrupts its interaction with hsp70 (86). However, Onate et al. (39) subsequently observed that hsp70 was not present or involved in specific recognition of a progesterone response element (PRE) by PR. Therefore, ER and PR may function differently with respect to hsp70 interaction. Thus, it will be especially important to determine the role of hsp70 in ER transcriptional activity or in the stabilization of ER/ERE interactions.

Effects of Associated Proteins on hER-ERE Interaction

Recent data indicate that some or all of the ER-associated proteins discussed above can influence the affinity and/or rate of ER-DNA complex formation. Two approaches were used, in conjunction with gel retardation analyses, to address this question: 1) removal of ER-associated components and 2) reconstitution experiments. Purifications schemes are outlined in **Fig. 1**. When analyzed by gel retardation (**Table 1**), maximal binding of hER to the vitellogenin ^{32}P-ERE (27 bp of natural vit. A2 gene sequence) occurred in the presence of all four hER-associated proteins (70,55,48,45 kDa) that were isolated by B-ERE chromatography (Purification scheme **C** in **Fig. 1**). This interaction is at least as good as the interaction between unpurified ER (CHO-ER nuclear extract) and the vit. ERE. Notably, the B-ERE eluate gives rise to two hER/ERE complexes. Subsequent removal of the 45 and 48 kDa proteins by chromatography of the B-ERE eluate on estradiol-Sepharose (E-Seph) in the presence of 0.7 M NaCl afforded hsp70-hER-p55 (70,66,55) complex that bound to ^{32}P-ERE with significantly reduced affinity (**Table 1**). The same hsp70-hER-p55 complex obtained by a single step purification of CHO-ER whole cell extract (WCE) on E-Seph (scheme **B, Fig. 1**), behaved similarly in gel shift experiments (**Table 1**). Proteins in the B-ERE/E-Seph nonadsorbed fraction (NA, scheme **C, Fig. 1**), which contained only the 45 and 48 kDa species, did not interact with the ^{32}P-labeled ERE in the absence of hER, at least under normal gel retardation conditions (**Table 1**). However, in the presence of a large excess of ^{32}P-ERE, a weak gel shift complex was observed, suggesting that at least one of the two proteins has some affinity for DNA, similar to the properties of the 45-kDa ER-ERE-binding protein reported by Mukherjee (46). When CHO-ER whole cell extract (WCE) was treated with ATP prior to E-Seph chromatography (scheme **A, Fig. 1**) to dissociate hsp70, the estradiol-eluted hER-p55 (66,55) complex displayed the weakest detectable interaction with the ^{32}P-ERE probe (**Table 1**), suggesting that hsp70 may play an additional role in stabilizing or faciliting ER-ERE interaction.

hER Reconstitution Studies

To test whether proteins that were dissociated from hER could be added back to reconstitute a more active complex, the E-Seph/ATP eluate (hER-p55, scheme **A, Fig. 1**) was first treated with an equimolar mixture of the 45 and 48 kDa proteins (E-Seph NA, scheme **C, Fig. 1**). As shown in **Table 1**, this combination enhanced the interaction of hER with ^{32}P-ERE. If hsp70 was added separately to the ER-p55 complex, a similar enhancement of hER-ERE interaction was observed, except that in this case a second, more retarded complex was also seen, suggesting the formation of an hsp70-hER-(p55?) complex. Finally, if all three proteins (45,48,70) were added to the hER-p55 complex, maximum hER-ERE formation was observed (**Table 1**), comparable to the original B-ERE purified material. In all experiments, a stoichiometric amount of each protein was added to a known, fixed amount of hER. Interestingly, a mutant ERE competitor (GGTCAnnnTG**CAC**), which has at least a ten-fold reduced affinity for fully complexed hER (65), competed significantly for the ER-p55-ERE complex, suggesting that one or more of the other associated proteins may contribute to ER-ERE specificity.

Measurement of the Rate of hER-ERE Association

The nature of the ER-ERE interaction was further characterized by a nitrocellulose filter binding assay. The filter binding assay is a simple method for quantitating DNA bound to protein based on the ability of nitrocellulose to bind proteins but not double-stranded DNA. The purpose of this assay was to quantitatively assess the association and dissociation rates of the differentially purified ER complexes as well as to examine the specificity of their interaction with the ERE. The association rate of each complex was measured by analyzing the DNA binding mixture at time points from 0-60 minutes following the addition of 32P-ERE. Analysis of the data demonstrated several points. First, as observed previously, the capacity of ER interaction with an ERE was significantly greater for the BERE complex than for either of the ligand affinity purified (ESeph or EATP) complexes. In addition, it appeared that under these experimental conditions the reactions had reached maximal ER-ERE interaction by as early as 10 minutes. Although an analysis of the initial rates, measured by the slope of the graphs prior to saturation, suggested that the rates of association may be different for the different ER complexes, the differences in the magnitude of the curves made this comparison difficult. Attempts to slow the rate of association rate by reducing the temperature to 4° C did not change to curves enough to allow comparisons either. Therefore analysis of the rate of dissociation of the complexes as well as Scatchard analysis was performed to clarify this issue.

Measurement of the Rate of ER-ERE Dissociation and Equilibrium Binding

A variation of the filter binding asay was performed to measure the rate of dissociation. The experiment was performed by incubating the BERE, ESeph and EATP complexes with ^{32}P-ERE. Once the reactions had clearly reached equilibrium (30 minutes), they were diluted ten-fold to quench the forward reaction. Subsequent to dilution, a portion of the reaction was removed and spotted onto nitrocellulose as the initial time point. Either TE control, ERE, mtERE or PRE was then added to the reaction and time points were monitored for 60 minutes. The results indicated that dissociation rates for the ER complexes were not significantly different. Therefore, the ER-associated proteins do not exert their influence on the rate of ER-ERE dissociation. To confirm these results, equilibrium saturation binding studies were carried out with the BERE, ESeph, and EATP complexes with ^{32}P-ERE. As expected from the association and dissociation rate data, no significant differences in equilibrium K_d constants ($K_d \approx 4 \times 10^{-9}$ M) were observed among these complexes. Only the absolute binding capacities (B_{max}) were different, as shown in **Fig. 2** and as summarized in **Table 1**.

Partially Purified ER Complexes Reveal Different DNA Bending Angles

In addition to the filter binding experiments just described, we measured, in collaboration with Ann Nardulli (University of Illinois-Champagne/Urbana) the extent of DNA bending induced by binding of the BERE, ESeph and EATP ER complexes to an ERE-containing DNA fragment (**Fig. 3**). The Nardulli lab had previously shown by circular permutation that partially purified, yeast-expressed ER as well as ER from an MCF-7 whole cell extract induced a bending angle of 65°. Analysis of the BERE, ESeph and EATP complexes also revealed a complex generating a bend angle of 65° (see **Fig. 4** and **Table 2**). Interestingly, the BERE complex formed three more slowly migrating complexes that were not detectable in the ESeph or EATP fractions (**Fig. 4**). These complexes generated bend angles of 74°, 92° and 96°, respectively (**Table 2**). Significantly, reexamination of earlier gels in which MCF-7 whole cell extract and CHO-ER nuclear extract were analyzed, revealed similar faint, more slowly migrating complexes (data not shown). The formation of all retarded complexes was inhibited effectively by an ERE competitor, but not by non-cognate DNA sequences (**Fig. 5**). These complexes were also supershifted by antibody directed against ER, indicating that ER is a part of the complex (**Fig. 5**). These results suggest that the associated proteins can influence the degree of DNA bending by ER.

Several conclusions can be drawn from the above results: 1) Maximal interaction of hER with vit. ERE requires at least two of the four ER-associated proteins (70,55,48,45) obtained from the B-ERE column. 2) A subpopulation of hER that is competent for DNA binding remains

associated with hsp70. Therefore, hsp70 may be required for high affinity ER/ERE interaction. 3) It is possible to sequentially dissociate and reconstitute complexes that consist of hER and at least some associated proteins. 4) Reconstitution studies suggest that the major contribution of these ER-associated proteins is to promote maximum interaction between hER and cognate ERE. 5) Circular permutation analysis results indicate that some of these proteins also contribute to the bend angle induced by ER interaction with EREs, which in turn may affect the rate of transcription initiation of responsive genes.

Summary and Model

Based on these results, as well as the published data of others regarding receptor-associated proteins (26, 87)(34), we propose a model (**Fig. 6**) in which unactivated p55-ER-hsp90/70 complex loses hsp90 following ligand binding; the resulting "activated" ER complex recruits or stabilizes the binding of two additional proteins (p45 & p48) when hER binds to ERE. The population of liganded hER which does not bind to ERE dissociates from hsp70. Other proteins (eg. hsp56, TFIIB) may participate in one or more of these steps. This model is not meant to be complete. Future work will focus on the identification and further characterization of these, and other, ER-associated proteins, especially in regard to their ability to influence DNA binding and/or transcriptional activation by ER. Of particularly interest is the mechanism(s) by which some estrogen antagonists (eg. OH-tam) appear to be able to promote DNA binding but then either fail to activate gene expression, or activate only a subset of genes, possibly by altered interaction of ER with accessory factors such as those described above or with other as yet unidentified intermediary factors.

Use of GST-ER Fusion Proteins To Isolate And Identify New Accessory Proteins.

Because important ER accessory or intermediary proteins may be tissue specific, limiting, or may bind less avidly to ER than those already isolated, a more sensitive *in vitro* method was needed to isolate additional ER associated proteins. Recently, we began to use a bacterially expressed GST-ER-LBD fusion protein (**Fig. 7**) to capture proteins from mammalian cell extracts that selectively associate with ER-LBD in the presence or absence of estradiol, hydroxytamoxifen, or ICI 182,780. With this method, a large excess of uncomplexed hER can be used as a trap for proteins that form heteromeric complexes with ER. This project is a powerful extension of the project described above for identifying proteins that bind/modulate ER activity. Bacterially expressed glutathione-S-transferase-ER fusion proteins (GST-ER) bound to glutathione-Agarose were used to adsorb cytosol or nuclear proteins derived from T47D, MCF-7, or HeLa cells. Notably, we have already isolated several ^{35}S-labeled proteins that bind to or modulate ER-LBD

selectively in the presence of estradiol or the complete antagonist ICI 182,780 (**Fig. 8**). The band observed at 160 kDa (p160) is almost certainly one of the RIP 140/160 proteins described recently by Myles Brown (88) and Malcolm Parker (89). The function of these proteins is not yet known, but it is noteworthy that they are nuclear proteins and that they bind to ER-LBD (AF2) only in the presence of an estrogen agonist such as estradiol. Also, p160 is present in multiple cell lines (eg. MCF-7 and T47D) (**Fig. 8**).

In addition to the agonist-specific capture of ^{35}S-p160 by GST-ER-LBD, two or possibly three other ^{35}S-labeled proteins (p44, p49, p55) were selectively retained by GST-ER-LBD only in the presence of ICI 182,780 (**Fig. 8**), suggesting that this pure estrogen antagonist promotes the interaction of ER with one or more proteins that may in some way mediate or reflect the antagonism of ICI 182,780. All three proteins observed in **Fig. 8** were present in both MCF-7 and T47D whole cell extracts, although the p44 band was less abundant in the MCF-7 cell extract. Additional experiments (not shown) showed that this ICI-mediated interaction could be competitively inhibited by estradiol. It is tempting to speculate that these proteins could be responsible either for the targeted degradation of ER that has been reported (55), or for sequestering ER and/or inhibiting productive interaction of the ER AF2 domain with the transcription initiation complex. To answer such questions, these proteins will be identified and their interactions mapped to the appropriate regions of ER. In vitro reconstitution experiments and in vivo blockade experiments should help elucidate their role in ER transcriptional activity.

Phosphorylation of hER-LBD

At least one protein retained selectively by GST-ER-LBD in the presence of estradiol is a kinase (probably serine/threonine) that can phosphorylate ER-LBD in an *in vitro* kinase assay (90) (**Fig. 9**). It is not yet clear whether this kinase is one of the ^{35}S-proteins observed in **Fig. 8** (ie. p160), or another protein that either does not label with ^{35}S-Methionine or is present at a level that is below the sensitivity of the method used. We are currently trying to isolate, identify and characterize this kinase. Because it is retained selectively by the GST-ER-LBD column in the presence of estradiol, it should be possible to elute and microsequence the kinase. When it is identified, we will study the hormone-mediated action of this kinase on ER transcriptional activity both in vitro and in vivo in appropriately transfected cell lines.

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FIGURE LEGENDS

Fig. 1. Purification scheme for hER isolated from CHO-ER whole cell extract (WCE).
A. Estradiol-Sepharose chromatography in the presence of ATP (E-Seph/ATP). **B**. Estradiol-Sepharose chromatography (E-Seph). **C**. DNA-affinity chromatography (B-ERE or B-ERE'E-Seph). (Note: when a sequential two step purification was performed, hER was purified on B-ERE agarose in the absence of estradiol). **D**. Immunoaffinity chromatography (H222-Seph). Reproduced from Landel et al. (91).

Fig. 2. Scatchard analysis of the ^{32}P-ERE binding affinity and capacity of hER complexes purified by B-ERE, E-Seph, and E-ATP affinity chromatography (see also **Fig. 1**). For details, see text.

Fig. 3. DNA bending vectors used in gel mobility shift assays. The DNA bending vectors used in circular permutation analysis have been previously described (23). ERE-containing plasmid was digested either with *Eco*RI, *Hing*III, *Eco*RV, *Nhe*I, or *Bam*HI to produce 430-bp DNA fragments containing a single consensus ERE at the indicated position. The restriction sites present in each fragment are shown at the top.

Fig. 4. DNA bending induced by purified hER complexes. hER complexes purified by B-ERE, E-Seph, and E-ATP affinity chromatography were incubated with ^{32}P-labeled DNA fragments RI, H, RV, N, and B (see **Fig. 3**). The protein-DNA mixtures were fractionated on an 8% nondenaturing polyacrylamide gel. The gel was then dried and radioactive bands were visualized by autoradiography. ER-DNA complexes are identified by number at the left of the figure.

Fig. 5. ERE Competition and antibody supershift analysis of hER-DNA complexes. Incubations and gel electrophoresis were carried out with purified hER complexes and the *Nhe*I-digested DNA fragment (**Fig. 3**), as described in the legend to **Fig. 4**. For supershift experiments, the ER-specific H222 monoclonal antibody was either added (+) or not added (-) to incubation mixtures prior to electrophoresis (left panel). Note that all four numbered complexes are shifted almost to the top of the gel in the presence of H222. For competition experiments, incubation mixtures contained either no competitor DNA (-), a 100-fold excess of unlabeled ERE (ERE+), or a 100-fold excess of unlabeled nonspecific DNA fragment (NS+) (right panel).

Fig. 6. Model for hER-associated proteins
This model is based on our current results and data reported by others for ER, PR and GR. Contact sites between hER and p45, p48 and p55 are unknown (indicated by "*"). Also, interactions among associated proteins are not resolved. D = DNA binding domain. S = steroid binding domain. Adapted from Landel et al. (91).

Fig. 7. Schematic representation of three GST-hER pGEX expression vectors. All three bacterial expression vectors were prepared in the laboratory of Peter Kushner (UCSF). Numbers refer to amino acids in the translated human ER. AF1 and AF2 refer to transcription activation functions one and two, originally described by Tora et al. (59) and Lees et al. (92). The GST-hER-LBD vector was used for all experiments described in this report.

Fig. 8. SDS-PAGE analysis of ^{35}S-Met-proteins retained on glutathione-agarose by GST-hER$_{282-595}$ (GST-hER-LBD). ^{35}S-Met-proteins present in whole cell extracts of metabolically labeled MCF-7 or T47D cells were incubated with glutathione-bound GST-hER-LBD or GST in the presence or absence of 1 μM estradiol (E2), 4-hydroxytamoxifen (OH-Tam) or ICI 182,780 (ICI182). Bound ^{35}S-proteins were eluted from the extensively washed beads with SDS sample buffer, fractionated by SDS-PAGE, and visualized by autoradiography. Migration of molecular weight markers is indicated at the left. The apparent molecular weights (kDa) of major retained proteins are indicated at the right. For details, see text.

Fig. 9. In vitro kinase assay of glutathione-agarose bound GST-hER-LBD following incubation with T47D whole cell extract. Unlabeled proteins present in T47D whole cell extract were incubated with glutathione agarose-bound GST-hER-LBD or GST in the presence or absence of 1 μM estradiol (E2), 4-hydroxytamoxifen (OH-Tam) or ICI 182,780 (ICI182). The beads were then washed extensively with loading buffer and incubated with ^{32}P-γ-ATP for 30 min at 30 C. Bound ^{32}P-labeled proteins were eluted from the extensively washed beads with SDS sample buffer, fractionated by SDS-PAGE, and visualized by autoradiography. Migration of molecular weight markers is indicated at the left. The position of GST-hER-LBD is indicated at the right. For details, see text.

Source of hER[1]	Proteins Present (kDa)[2]	Relative DNA Binding
CHO-ER Nuc. Ext.	total nuclear proteins	+++
B-ERE	70, 66, 55, 48, 45	++++
B-ERE→E-Seph EL	70, 66, 55	++
B-ERE→E-Seph NA	48, 45	-
E-Seph	70, 66, 55	++
E-Seph/ATP	66, 55	+

Table 1. Summary and properties of ER-associated proteins isolated by several chromatographic techniques (see also **Fig. 1**).

[1]Three methods were used to isolate hER: Site-specific DNA-affinity chromatography (B-ERE), Estradiol-sepharose affinity chromatography (E-Seph), Estradiol-sepharose chromatography in the presence of ATP (E-Seph/ATP). A two step purification using B-ERE followed by E-Seph was also used. The eluate (B-ERE→E-Seph EL) and nonadsorbed (B-ERE→E-Seph NA) are indicated above.

[2]The identity of the proteins indicated in the table are: 70 = hsp70; 66 = hER, 55 = PDI, 48 and 45 are unidentified.

[3]NA = not available

Source	Complex	Mean Bend Angle	Std. Error
BERE	1	61.8	0.88
	2	65.6	0.58
	3a	74.5	1.88
	3b	93.0	1.64
	4	96.8	2.50
ESeph	1	61.5	0.88
	2	65.0	0.35
EATP	1	62.1	1.02
	2	64.0	0.53

Table 2. Bend Angles of Partially Purified hER complexes. Complexes 1-4 are shown in Figs. 4 & 5. Complex 1 represents the least retarded (lowest) hER-[^{32}P]ERE band.

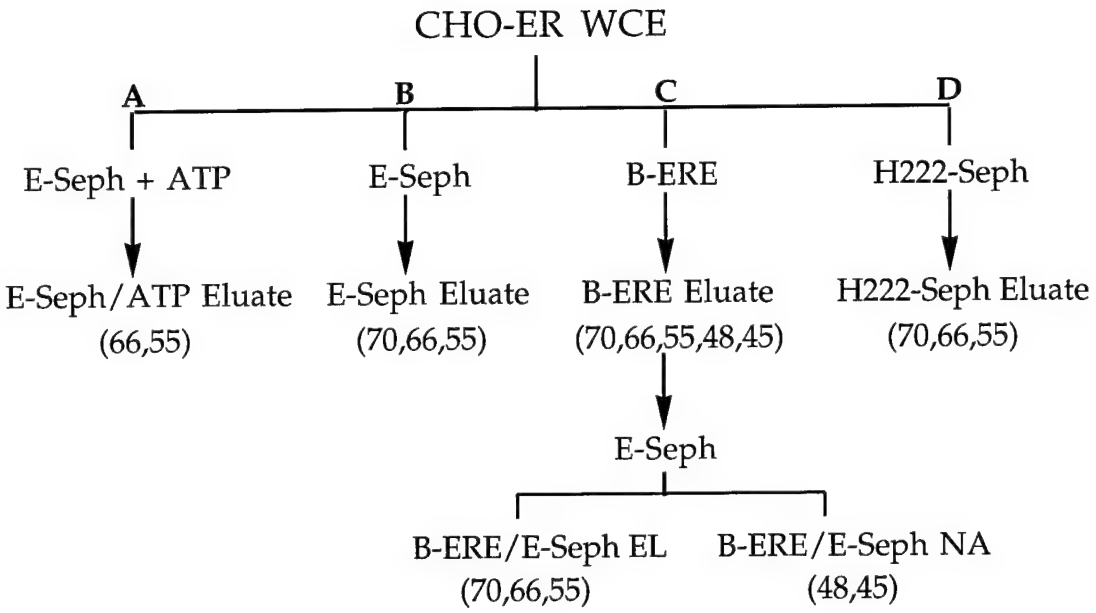

CHO-ER WCE
A
B
C
D
E-Seph + ATP
E-Seph
B-ERE
H222-Seph
E-Seph/ATP Eluate
(66,55)
E-Seph Eluate
(70,66,55)
B-ERE Eluate
(70,66,55,48,45)
H222-Seph Eluate
(70,66,55)
E-Seph
B-ERE/E-Seph EL
(70,66,55)
B-ERE/E-Seph NA
(48,45)

Fig. 1

Scatchard Analysis

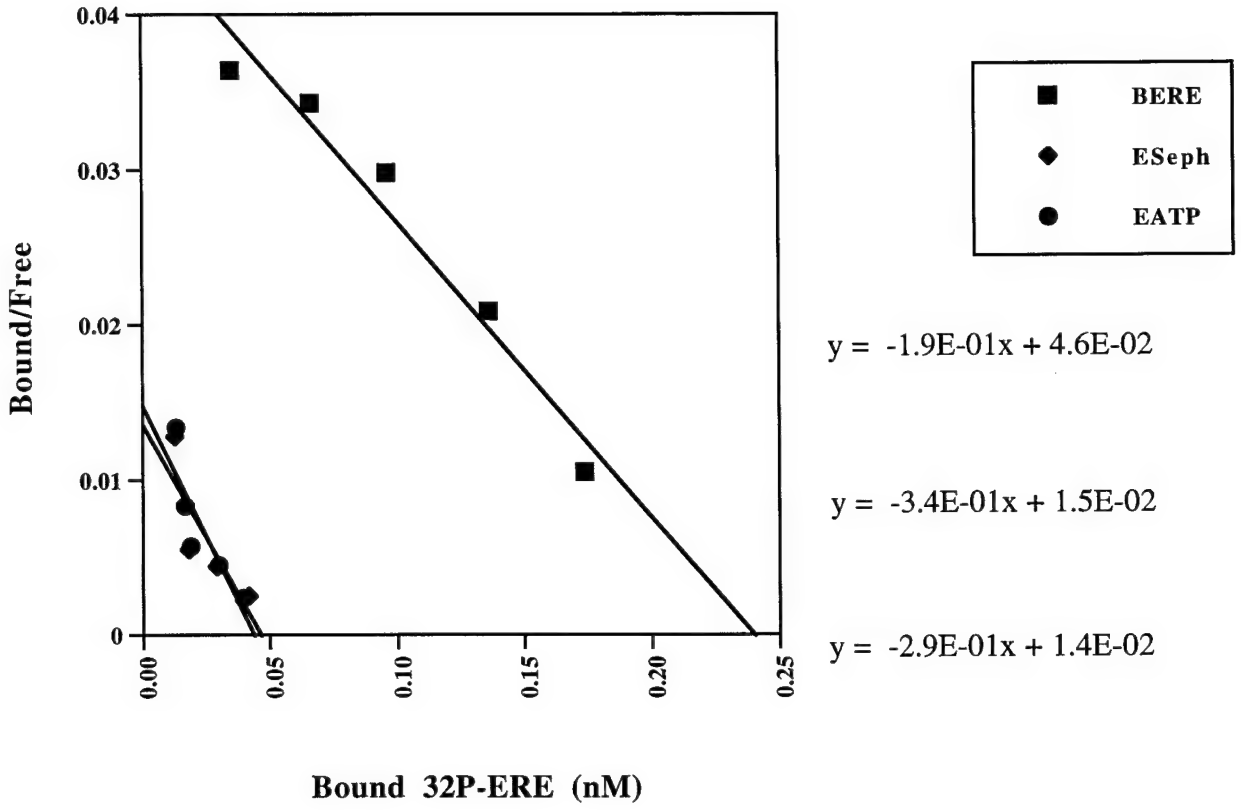
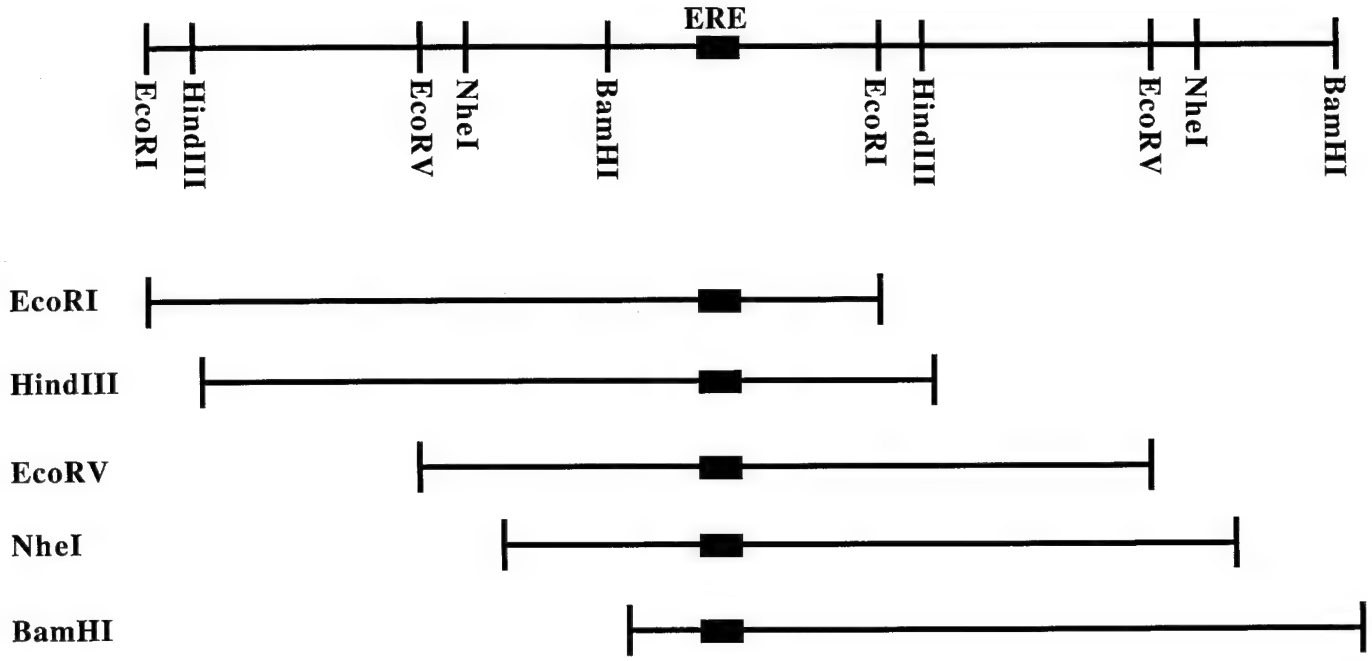


Fig. 2

Circular Permutation Analysis



$$\mu M/\mu E = \cos [\alpha/2]$$

μ = migration of the ER-DNA complex divided by the migration of the corresponding uncomplexed DNA where the ERE is near the middle (M) or the end (E) of the fragment

Fig. 3

Circular Permutation Analysis

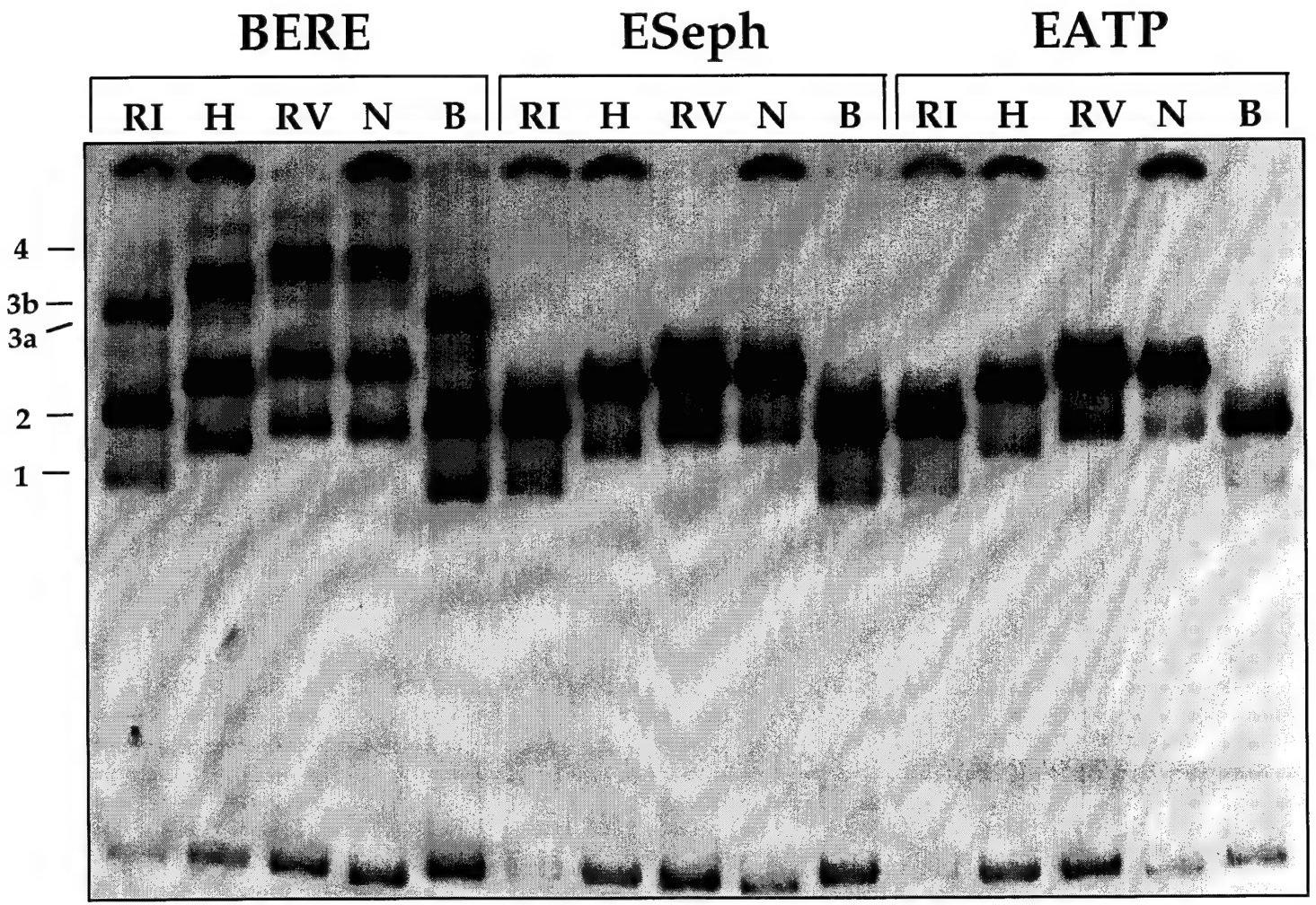


Fig. 4

Competition and Supershift Analysis

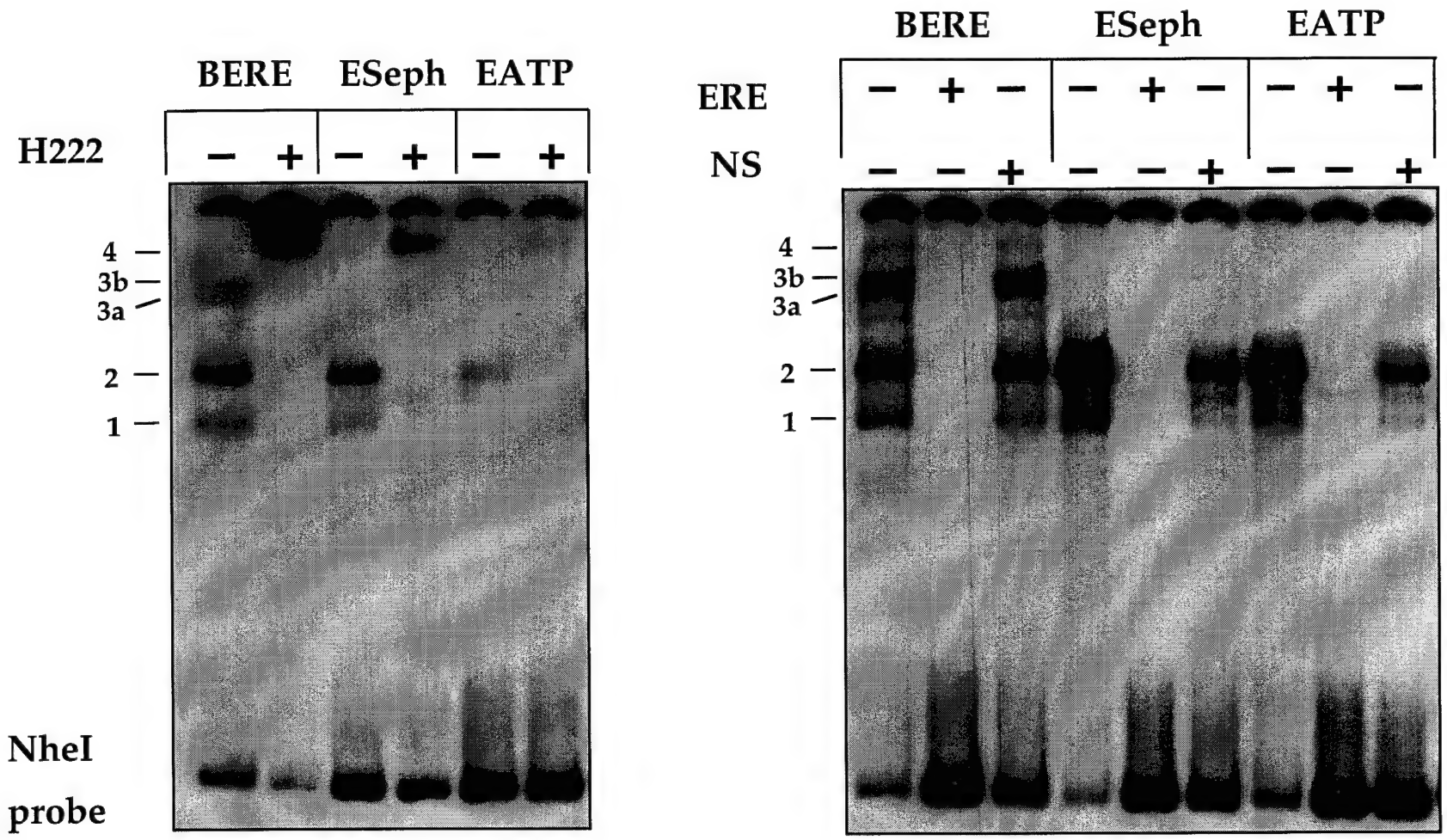


Fig. 5

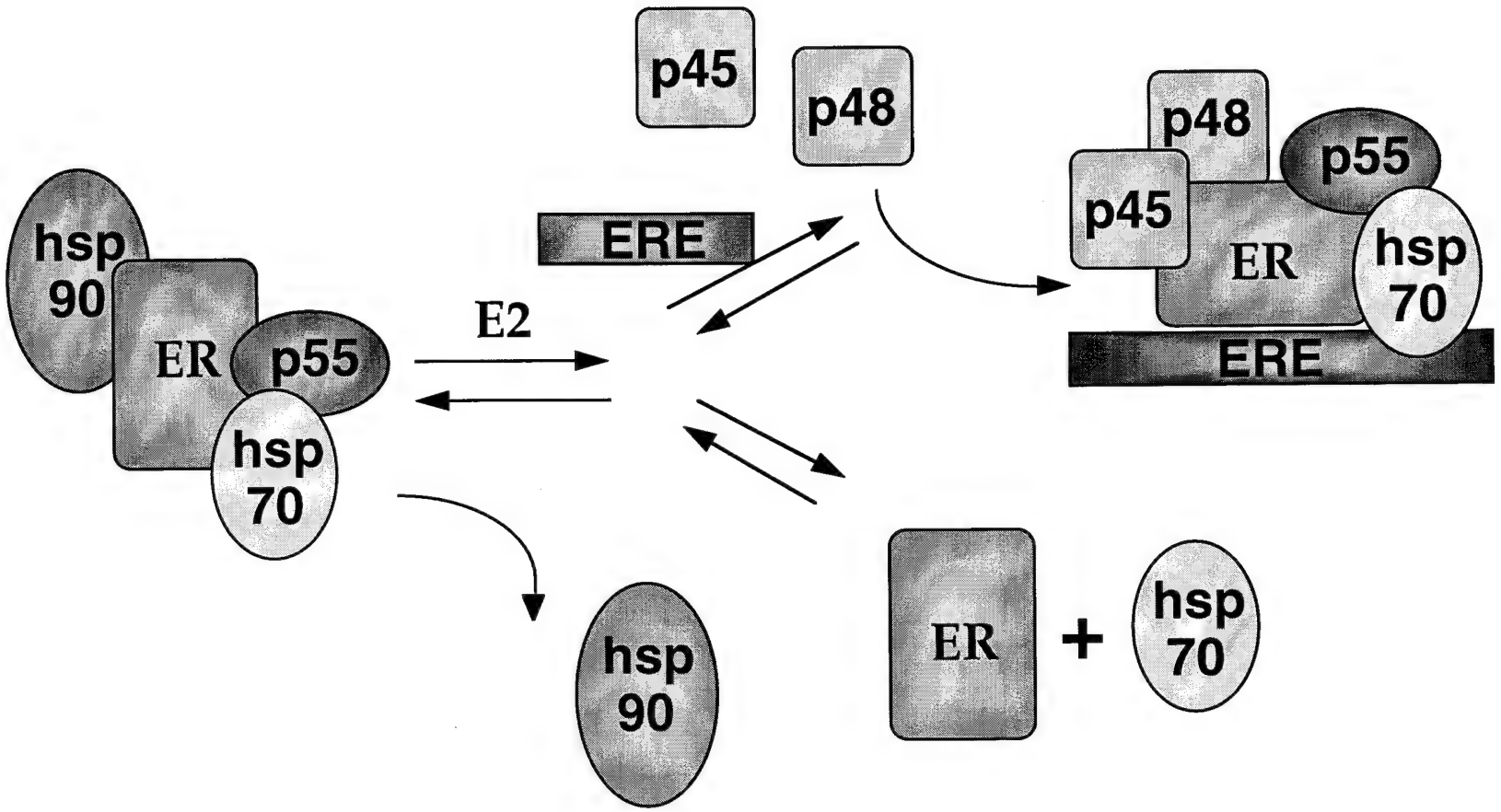

p45
p48
p48
p55
p45
ER
hsp
70
ERE
hsp
90
ER
p55
E2
hsp
70
ERE
hsp
90
ER
+
hsp
70

Fig. 6

GST-hER
expression vectors

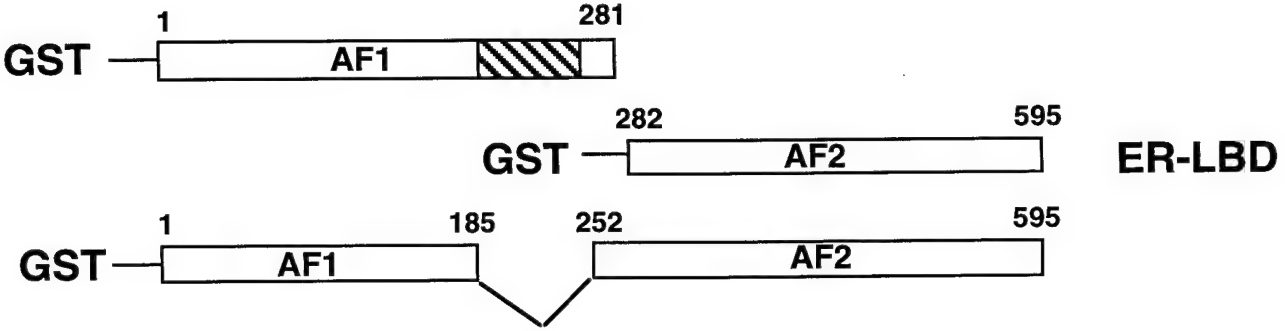


Fig. 7

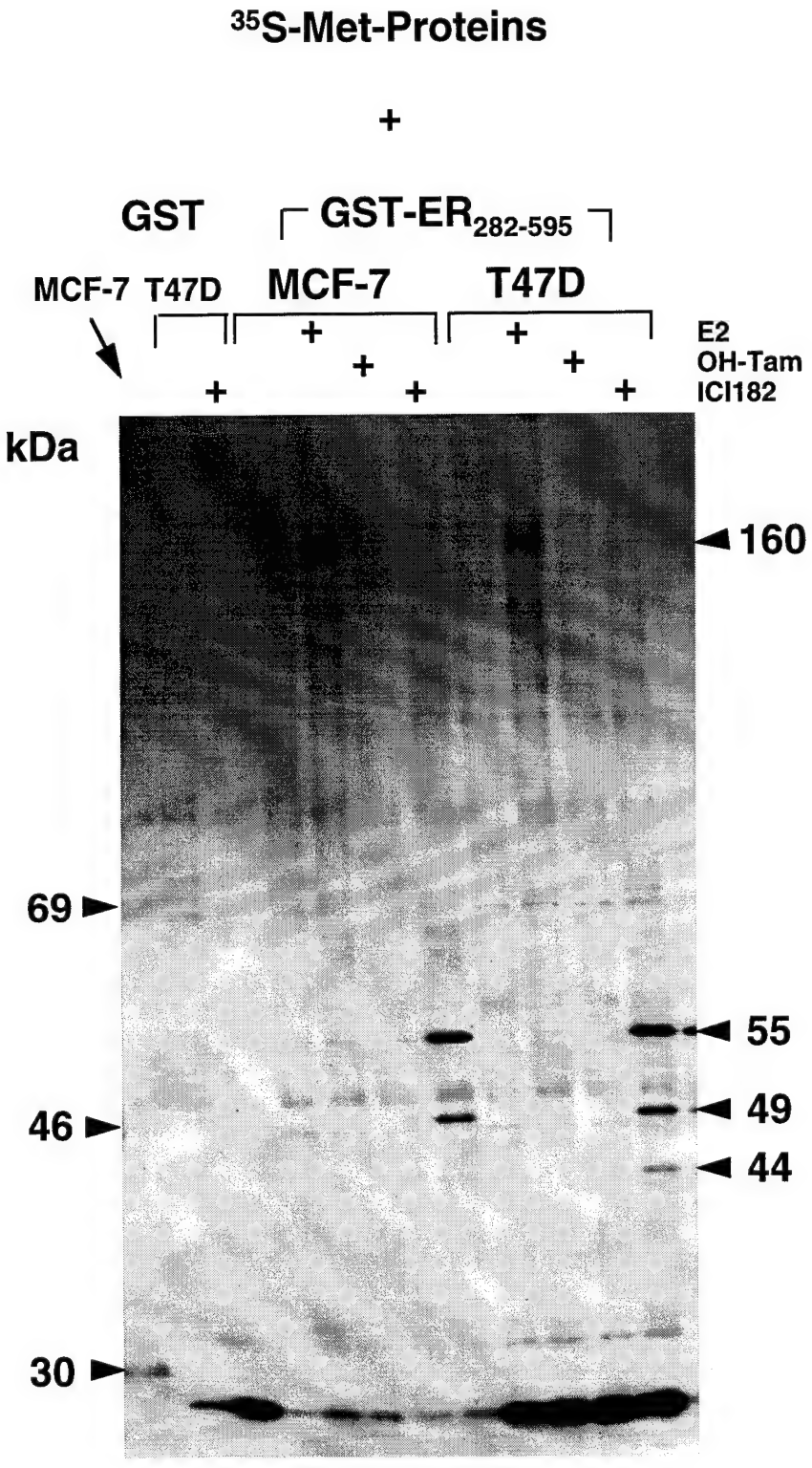

35S-Met-Proteins
+
GST
GST-ER282-595
MCF-7 T47D
MCF-7
T47D
E2
OH-Tam
ICI182
kDa
160
69
55
49
46
44
30

Fig. 8

In vitro Phosphorylation of GST-ER-LBD

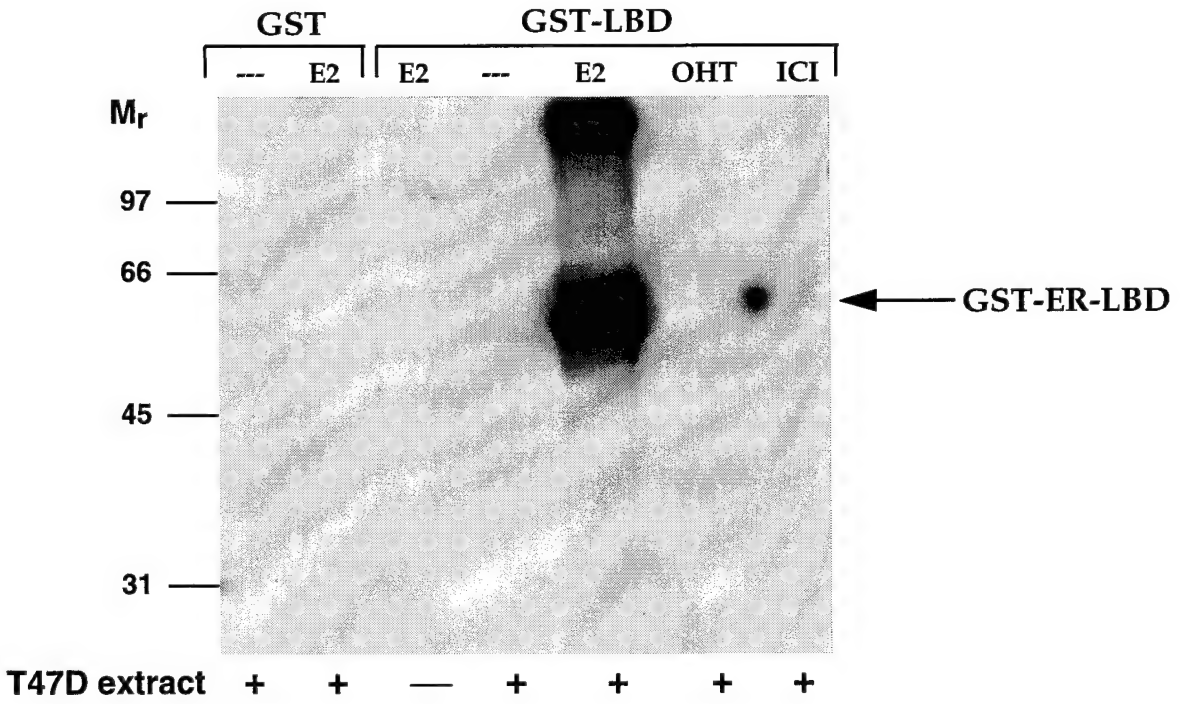


Fig. 9

The Interaction of Human Estrogen Receptor with DNA Is Modulated by Receptor-Associated Proteins

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To better define the role of accessory proteins as mediators of estrogen receptor (ER) function, we have used immuno-, steroid-, and site-specific DNA-affinity chromatography to identify and characterize proteins that associate with ER in extracts from ER-expressing Chinese hamster ovary (CHO-ER) cells. Two associated proteins [70 and 55 kilodaltons (kDa)] were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis silver stain analysis of all three column eluates. Two additional proteins (45 and 48 kDa) were preferentially retained by the ER-specific DNA affinity column. The 70-kDa protein was subsequently identified by Western blot analysis as a heat shock protein (hsp70). The 55-kDa protein was identified by N-terminal microsequencing as a member of the protein disulfide isomerase family. The 48- and 45-kDa proteins remain unidentified. To determine the possible differential effects of estrogen agonists and antagonists on human (h) ER interaction with these proteins, CHO-ER cells were labeled with [^{35}S]methionine in the presence of estradiol, 4-hydroxytamoxifen (OH-Tam; partial agonist/antagonist), or ICI 182,780 (complete antagonist). None of these ligands altered the pattern of associated proteins when hER complexes were isolated by adsorption to the vitellogenin A2 estrogen response element (ERE). However, when hER was isolated by immunoadsorption, a reduction in the level of associated hsp70 was observed following treatment with estradiol or OH-Tam, compared to no treatment or treatment with ICI 182,780. By gel retardation analysis, maximal interaction of affinity-purified hER with ERE occurred in the presence of all four associated proteins. Removal of the 48- and 45-kDa proteins and/or hsp70 resulted in a decrease in hER/ERE association, which could be restored by the addition of purified hsp70 and/or a mixture of the 48- and 45-kDa proteins. The increased stability of the restored complex was due primarily to an increase in the association rate of hER with ERE. These results suggest that accessory proteins may be required for maximal ER interaction with EREs and that estrogens and estrogen antagonists may promote differential retention of hsp70 in the presence or absence of a specific ERE. (Molecular Endocrinology 8: 1407–1419, 1994)

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Molecular Endocrinology

INTRODUCTION

Steroid hormones regulate gene expression via the interaction of specific intracellular receptor proteins with the genome. As a consequence, DNA synthesis is altered, as well as the synthesis of specific RNAs and proteins that mediate cell proliferation, differentiation, and physiological function and development in diverse tissues and species. The cloning and molecular analysis of the known steroid receptors have led to the definition of common functional domains and a proposed mechanism by which they interact with responsive genes (1). Thus, all of the steroid receptors, including the estrogen receptor (ER), are activated by one or more ligands and bind with high affinity and specificity to short *cis*-acting DNA sequences referred to as hormone response elements. Interaction of steroid-receptor complexes with responsive genes can result in either induction or repression of transcription, depending upon the target gene and tissue.

The participation of accessory proteins, both before and after hormonal activation, has been the subject of much investigation (2). At least three members of the heat shock protein (hsp) family have been identified as putative accessory proteins by virtue of their associa-

tion with several receptors *in vitro*. One of these, hsp90, has been implicated in the *in vitro* stabilization of the inactive form of receptors for glucocorticoids (GR), progestins (PR), and estrogens (ER) (3). In support of the hypothesis of an essential role for hsp90, recent experiments in which expression of the hsp90 gene was conditionally regulated in *Saccharomyces cerevisiae* demonstrated that reduced levels of hsp90 severely compromised GR transcriptional activity (4). ER activity was less affected in this model. In contrast, members of the thyroid (5) and retinoid (6) receptor family do not appear to associate with hsp90, but rather are synthesized in an active form that is able to bind to hormone response elements *in vivo* in the absence of ligand. hsp56, which was recently identified as an immunophilin, also appears to be part of the unactivated complex of several steroid receptors (7) and may be able to modify the transcriptional activity of some receptors (*e.g.* PR) in response to immunosuppressants such as FK506 (8). Another heat shock protein, hsp70, has been shown to bind to both PR and GR in the absence of hormone, but unlike hsp90, this association appears to be maintained, at least in part, in the activated receptor complex after hormone treatment (9). A recent study using baculovirus overexpressed human GR in *Spodoptera frugiperda* cells suggested that hsp70 is associated with the GR-glucocorticoid response element complex (10).

An additional class of receptor-associated proteins are those that interact with the activated receptor complex. Thus, a 55-kilodalton (kDa) nuclear accessory factor has been reported to be essential for maximal binding of the vitamin D receptor to the vitamin D response element from the human osteocalcin promoter (11). Similarly, a 65-kDa factor termed triiodothyronine receptor-auxiliary protein (TRAP), which exhibits limited independent DNA binding, has been shown to enhance TR binding to DNA (12). In addition, a 110-kDa receptor accessory factor (RAF) has been observed to potentiate the DNA binding activity of the androgen receptor (13). The nonhistone high mobility group chromatin protein, HMG-1, has been shown to substitute for an unidentified factor present in partially purified progesterone receptor fractions that is responsible for promoting PR-DNA binding (14).

The role of accessory proteins in mediating the activities of the ER remains largely unknown. Although ER has been shown to associate with hsp90 as well as hsp56 *in vitro* (15), the significance of these interactions *in vivo* remains unclear. Similarly, while ER has been shown to bind TFIIB *in vitro* via transcriptional activation function 2 (TAF-2) (16), it is likely that still other general, as well as receptor- and cell-specific, factors exist that modulate the activity of this region and TAF-1 of ER. One approach to better define the existence, identity, and role of ER-associated proteins is to isolate and analyze receptor complexes from cell extracts. For this purpose, we used immunoaffinity, steroid affinity, and site-specific DNA-affinity chromatography to identify proteins that copurify with the human ER in high salt (0.4 M NaCl) extracts from MCF-7 human breast cancer cells (17) and Chinese hamster ovary cells stably transfected with human ER (CHO-ER cells)(18). By using lysis conditions that disrupt the association of hER with hsp90 and p56, we were able to identify proteins that interact with activated hER. We then investigated the effects of estrogen agonists and antagonists on the association of hER with the identified accessory proteins. Finally, we determined the effects of these associated proteins on the interaction of hER with the classical estrogen response element (ERE) from the vitellogenin A2 gene, both by selective removal of associated proteins and by reconstitution experiments.

RESULTS

Chromatographic Isolation of hER-Associated Proteins

ER-associated proteins were isolated from high salt extracts of CHO-ER cells and MCF-7 cells by steroid-affinity (E-Seph), site-specific DNA-affinity [biotinylated-ERE (B-ERE)], and immunoaffinity (H222-Seph) chromatography (Fig. 1). CHO-k1 cell extracts, which do not contain ER, were used as negative controls. Analysis of the eluted proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a similar, but not identical, pattern of associated proteins for each technique (Figs. 2 and 3). In each case, the expected 66-kDa hER was observed in silver stained gels (Fig. 2) as well as on Western blots (Fig. 3, panel B). Several minor degradation products of hER were also observed on the Western blot. In addition to hER, a 70-kDa protein was present in eluates from all three columns (Fig. 2). Western blot analysis identified this species as a member of the hsp70 family of proteins

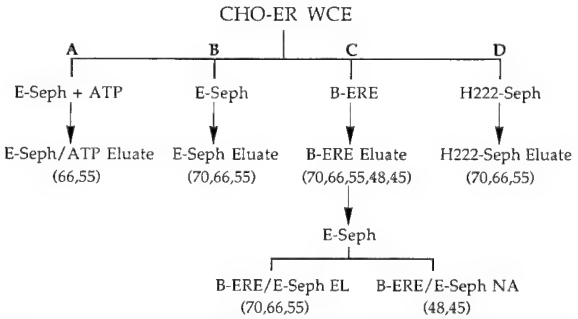

Fig. 1. Purification Scheme for hER Isolated from CHO-ER Whole Cell Extract (WCE)

A, Estradiol-Sepharose chromatography in the presence of ATP (E-Seph/ATP). B, Estradiol-Sepharose chromatography (E-Seph). C, DNA-affinity chromatography (B-ERE or B-ERE→ E-Seph). (Note: when a sequential two-step purification was performed, hER was purified on B-ERE agarose in the absence of estradiol.) D, Immunoaffinity chromatography (H222-Seph). Details of each chromatographic technique are described in *Materials and Methods*.

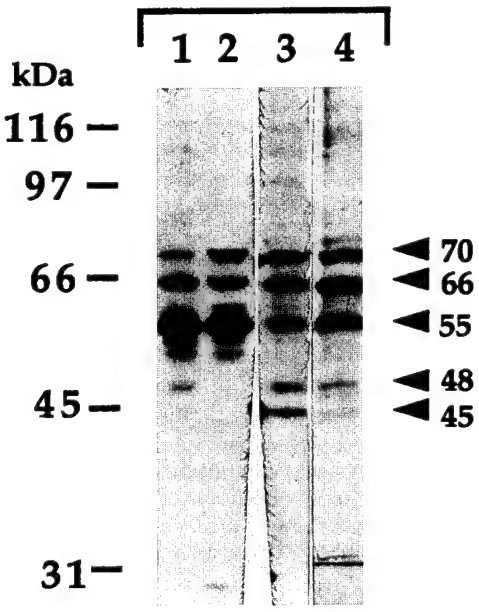

Fig. 2. Silver Stain Analysis of hER-Associated Proteins Identified by Three Chromatographic Techniques

hER complexes were purified by E-Seph/ATP (lane 1), E-Seph (lane 2), B-ERE (lane 3), or H222-Seph (lane 4). CHO-ER WCE was adjusted to contain the appropriate buffer (see *Materials and Methods*) for each chromatographic technique and incubated for 1 h at 4 C with the indicated adsorbents. Bound receptor complexes were then eluted and analyzed by 10% SDS-PAGE. Proteins were visualized by staining with silver. Migration of the mol wt markers are indicated on the *left*. The position and estimated mol wt of the eluted proteins are indicated on the *right*.

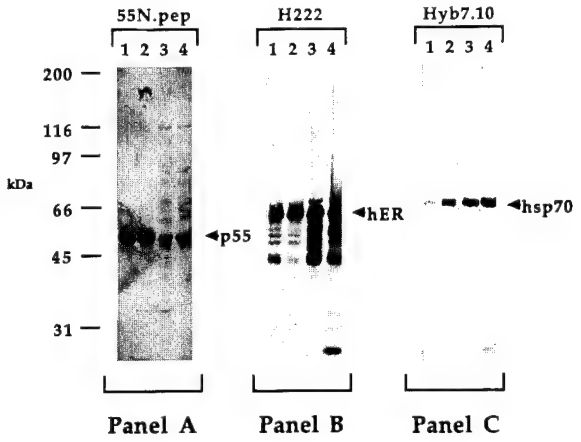

Fig. 3. Western Blot Analysis of hER-Associated Proteins Identified by Three Chromatographic Techniques

hER complexes were purified as described in Fig. 2 by E-Seph/ATP (lane 1), E-Seph (lane 2), B-ERE (lane 3), or H222-Seph (lane 4). Eluted proteins were then separated by 10% SDS-PAGE and electrotransferred to nitrocellulose. Blots were probed with antibodies directed against PDI (panel A), hER (panel B), or hsp70 (panel C). Migration of the mol wt markers is indicated on the *left*. The position and identity of the proteins are indicated to the *right* of each panel.

(Fig. 3, panel C). Treatment of CHO-ER whole cell extract with ATP before purification of hER by E-Seph chromatography generated an hER complex (E-Seph/ATP) with significantly reduced levels of associated hsp70, consistent with the reported behavior of hsp70 proteins (19).

An additional 55-kDa protein (p55; Fig. 2) coeluted with hER purified by all three methods. To identify this protein, the hER/p55 complex (100–200 pmol of hER) was isolated by E-Seph chromatography (20) from either CHO-ER or MCF-7 whole cell extracts. Eluted proteins were separated by SDS-PAGE, transferred to Immobilin-P, and stained with Ponceau S. The 55-kDa band was then excised and treated *in situ* with 3-bromo-3-methyl-2-(nitrophenylmercapto)-^{3}H-indole (BNPS-skatole), a reagent that cleaves proteins at the C-terminal end of tryptophan residues (21). Cleaved fragments were eluted from the membrane, separated by SDS-PAGE, transferred to Immobilon-P, and visualized by staining with Coomassie brilliant blue. Selected fragments, as well as the full-length protein, were then excised and sequenced directly from the membrane (see *Materials and Methods* for sequence data). A comparison of the obtained peptide sequences with sequences found in the Swiss-Prot data base (GenBank) indicated that p55 was a member of the protein disulfide isomerase (PDI) family (22), although six amino acids of the hamster p55 differed from the reported human PDI sequence. To determine whether these differences were related to the source of p55 (CHO-ER cells), p55 was isolated from MCF-7 cells and sequenced by the same method. MCF-7 p55 sequence was identical to the known human PDI sequence. A polyclonal rabbit antibody specific for the N-terminal region (residues 1–22) of hamster p55 was generated and used on Western blots to identify the presence of p55 in subsequent experiments (Fig. 3A). Although trace amounts of hsp70 and p55 were observed in E-Seph eluates when CHO-k1 extracts were substituted for CHO-ER extracts, their retention was significant only when ER was present (data not shown).

Two additional proteins that migrated at 48- (p48) and 45-kDa (p45) were observed in eluates containing hER purified by B-ERE chromatography (Fig. 2, lane 3). Although proteins of similar mol wt were sometimes observed in H222-Seph eluates (Fig. 2, lane 4), these results were variable and it is unclear whether these proteins represent the same species observed in B-ERE eluates. Regardless, the association of p48 and p45 with hER was clearly enhanced when hER was bound to an ERE. These results suggest that this association is promoted or stabilized by ER-ERE interaction. Identification of these two proteins is in progress.

Effect of Agonist and Antagonist on hER-Associated Proteins

To examine possible differential effects of estrogen agonists and antagonists *in situ* on the interaction of

hER with associated proteins, cells were metabolically labeled with [^{35}S]methionine during treatment with estradiol (E_2), the pure antagonist ICI-182,780 (ICI-182), or the partial antagonist OH-Tam. The cells were subsequently harvested and lysed by three freeze-thaw cycles. hER complexes were isolated from the extracts by either H222-Seph or B-ERE chromatography (Figs. 4 and 5). None of the tested ligands affected the stoichiometry of hER-associated proteins when hER was purified by B-ERE chromatography (Fig. 4). hER, p55, p48, and p45 observed previously by silver stain (Fig. 2, lane 3) were also seen in the autoradiogram of the dried polyacrylamide gel (Fig. 4, panel 1). The absence of a ^{35}S-met-hsp70 band is presumably due to the low turnover rate of hsp70 (23). However, hsp70, as well as hER and p55, were readily detected by Western blot analysis (Fig. 4, panel 2).

In contrast to the B-ERE chromatography results, hER complexes isolated by H222-Seph chromatography showed a significant reduction in the amount of associated hsp70 following treatment of CHO-ER cells *in situ* with either E_2 or the partial agonist OH-Tam (Fig. 5B). However, dissociation of hsp70 did not occur in the absence of ligand or when cells were treated with the complete antagonist ICI-182 (Fig. 5B). These results suggest that hsp70 may be required for high affinity ER-ERE binding and that a subpopulation of ER that is competent for DNA binding remains associated with hsp70. The additional immunoreactive species observed in the Western blot shown in Fig. 5 represent heavy and light chain immunoglobulin G (IgG) contaminants from the immunoadsorbent that were released during elution.

Interactions of hER Complexes with DNA

The electrophoretic mobility shift assay (gel shift) was used to examine the effect of ER-associated proteins on the stability and/or rate of ER-ERE complex formation. Both the selective removal of hER-associated proteins as well as the reconstitution of hER complexes were used to address this question. To isolate p48 and p45, hER from unlabeled CHO-ER whole cells extracts was purified by B-ERE chromatography, affording the complex described earlier (hsp70/hER/p55/p48/p45). This complex was further fractionated by E-Seph chromatography, in the presence of high salt (0.7 M NaCl) and in the absence of DNA, to release p48/p45 from bound hER/hsp70/p55 (Fig. 1). The p48/p45 mixture present in the nonadsorbed fraction (B-ERE→E-Seph NA) was used in subsequent reconstitution experiments. To confirm the selective removal of p48 and p45 following E-Seph chromatography, the bound and nonadsorbed proteins from each chromatographic step were visualized by silver stain on SDS-PAGE gels (data not shown). Endogenously expressed hsp70 proteins, purified from *Saccharomyces cerevisiae* by ATP-agarose chromatography to greater than 90% of the total protein (24), were also used for reconstitution experiments. For all gel shift studies, partially purified hER preparations were standardized for final hER content by contolled-pore glass bead (CPG) assay (25).

As shown in Fig. 6B (lane 1), the most intense [^{32}P]-ERE/ER complex was observed in the presence of all four hER-associated proteins that were isolated by B-ERE chromatography. This interaction was as stable

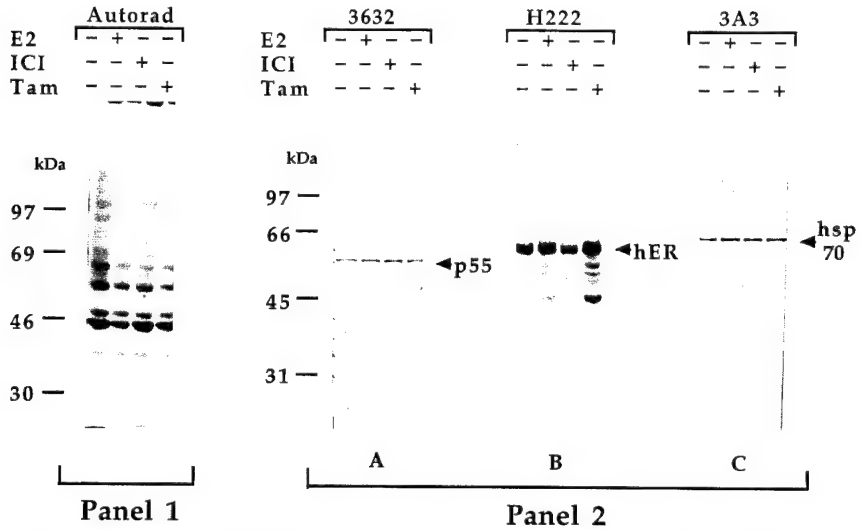

Fig. 4. Comparison of hER-Associated Proteins Isolated by DNA-Affinity Chromatography after Treatment of CHO-ER Cells with No Ligand, Estradiol, ICI-182,780, or OH-Tam during [^{35}S]Methionine Labeling

CHO-ER cells were labeled with [^{35}S]methionine in the presence of the ligands indicated in the figure, and hER was isolated by extraction in the presence of 0.4 M NaCl. hER complexes were purified from the extract by adsorption to B-ERE and analyzed by SDS-PAGE. Panel 1 is an autoradiogram of ^{35}S-labeled proteins present in the complex. Western blots were probed with antibodies directed against PDI (panel 2A), hER (panel 2B), or hsp70 (panel 2C) and visualized colorimetrically. Migration of the ^{14}C-labeled mol wt markers for the autoradiogram is indicated to the *left* of panel 1. Migration of the mol wt markers for the Western blot is indicated to the *left* of panel 2. The position and identity of the proteins in panel 2 are indicated on the *right*.

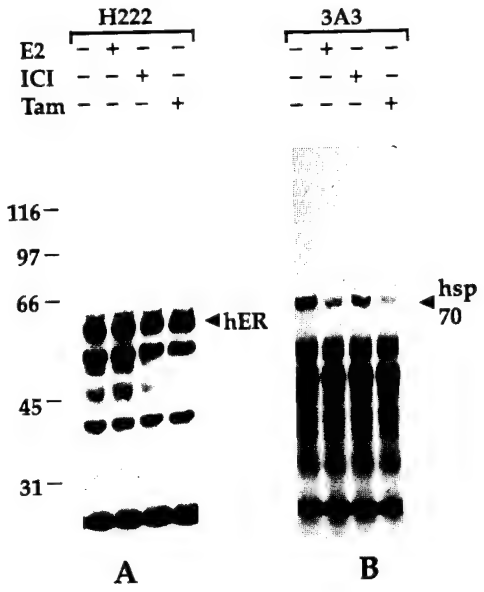

Fig. 5. Western Blot Analysis of hER-Associated Proteins Isolated by Immunoaffinity Chromatography after Treatment of CHO-ER Cells with No Ligand, Estradiol, ICI-182,780, or OH-Tam during [^{35}S]Methionine Labeling

CHO-ER cells were labeled with [^{35}S]methionine, and hER was isolated by extraction in the presence of 0.4 M NaCl. hER complexes were purified from the extract by adsorption to H222-Seph and analyzed by SDS-PAGE. Western blots were probed with antibodies directed against hER (panel A), or hsp70 (panel B), and visualized colorimetrically. Migration of the mol wt markers is indicated on the *left*. The position and identity of the proteins are indicated on the *right*.

as that observed for unpurified ER from CHO-ER nuclear extracts (Fig. 6A, lane 1). Subsequent removal of p48/p45 yielded a [^{32}P]ERE/ER complex (hsp70/hER/p55) with reduced intensity (Fig. 6B, lane 4). A similar result was obtained when this same complex was isolated directly from CHO-ER whole cell extract by E-Seph chromatography (Fig. 6A, lane 4). Proteins present in the B-ERE→E-Seph NA fraction described above (p48/p45) did not interact with [^{32}P]ERE (Fig. 6B, lane 7). Dissociation of hsp70 by treatment of CHO-ER whole cell extract with ATP before purification of hER by E-Seph chromatography (E-Seph/ATP) afforded the least intense [^{32}P]ERE/ER complex (hER/p55) (Fig 6C, lane 1). These results suggest that either p48, p45, or both, as well as hsp70, may play a role in stabilizing or facilitating the ER-ERE interaction. Antibody supershift experiments were performed with an ER-specific monoclonal antibody to demonstrate that hER was present in all [^{32}P]ERE gel shift complexes (Fig. 7).

To test whether proteins that were dissociated from hER could be added back to reconstitute a more stable hER/ERE complex, the hER complex (hER/p55) present in the E-Seph/ATP eluate was first treated with an amount of p48/p45 (B-ERE→E-Seph NA) that was stoichiometrically equivalent to the levels observed in the initial B-ERE eluate. This combination enhanced the intensity of the shifted [^{32}P]ERE/hER complex (Fig. 6C, lane 4). When purified hsp70 was added separately to hER in the E-Seph/ATP eluate, a similar enhancement of the [^{32}P]ERE/hER band was observed, and in this case a second, more slowly migrating, complex also appeared (Fig. 6C, lane 7). The most intense [^{32}P]ERE/hER band, comparable to the original B-ERE-purified hER complex, was observed when all three proteins (p45, p48, and hsp70) were added to E-Seph/ATP-purified hER (Fig. 6C, lane 10). These results demonstrate that it is possible to sequentially dissociate and reconstitute complexes that consist of hER and at least some *in vitro* associated proteins. Furthermore, the data also suggest that maximal interaction of hER with the vitellogenin ERE requires at least two of the four proteins obtained from B-ERE chromatography.

The gel shift assay was also performed in the presence of a 100-fold excess of unlabeled vitellogenin A2 ERE or a mutant ERE (26), bearing a 2 base pair inversion in the second half of the palindrome (TG**CA**CT), to determine the specificity of [^{32}P]ERE/hER interactions (Fig. 6). All [^{32}P]ERE/hER complexes were effectively competed with excess ERE. Interestingly, removal of the p48/p45 fraction, or hsp70, led to an enhanced sensitivity of [^{32}P]ERE/hER to competition by the mutant ERE, suggesting that the hER-associated proteins may contribute to the specificity of the hER-ERE interaction. The significance of this result is being investigated in more rigorous competition experiments.

A modification of the gel shift assay was used to measure the rate of hER-ERE interaction in the presence of added associated proteins (Fig. 8). The minimal hER complex (E-Seph/ATP eluate; hER/p55) afforded a weak [^{32}P]ERE/hER band at 15 min (Fig. 8). Addition of the p48/p45 fraction to this complex gave rise to a [^{32}P]ERE/hER band of comparable intensity after only 3 min (Fig. 8). Similarly, the addition of hsp70 also enhanced the rate of [^{32}P]ERE/ER association, as well as generating the doublet band pattern described above (Fig. 8). Finally, when both the p48/p45 fraction and hsp70 were added, the [^{32}P]ERE/hER complex was observed at the earliest measured time point (Fig. 8). Results similar to those just described for complete reconstitution were also observed for the hER complex isolated by a single step purification on B-ERE (data not shown). These results suggest that the hER-associated proteins can contribute to the rate of hER-ERE interaction.

DISCUSSION

We have used immunoaffinity, steroid-affinity, and site-specific DNA-affinity chromatography to isolate proteins that associate with ER in MCF-7 and CHO-ER cell extracts (Fig. 1). By lysing cells under conditions that disrupt the association of hER with hsp90 and p56 (0.4 M NaCl), we were able to identify proteins that interact with activated hER. All three approaches revealed the

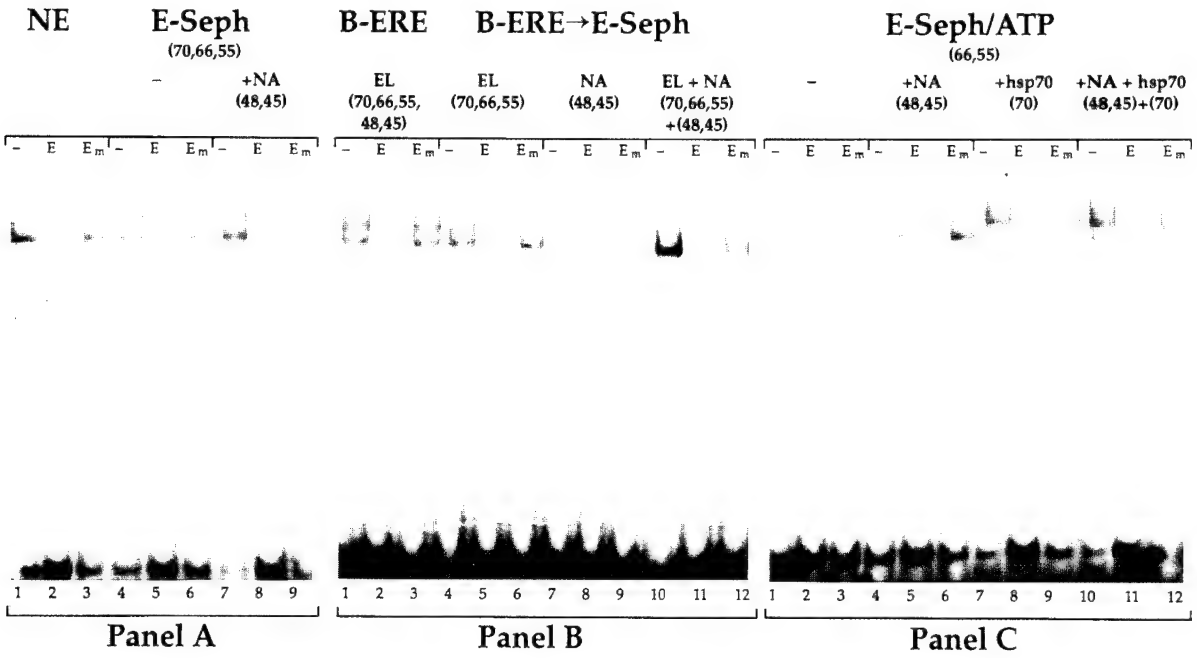

Fig. 6. Electrophoretic Mobility Shift Analysis of hER/^{32}P-ERE Interactions in the Presence or Absence of hER-Associated Proteins

hER complexes were incubated for 30 min at 25 C with ^{32}P-labeled vitellogenin A2 ERE (27-mer) and electrophoresed on a 5% nondenaturing polyacrylamide gel. The source and composition of each mixture are outlined in Fig. 1. E and E_m indicate the addition, respectively, of excess unlabeled vitellogenin A2 ERE (AGGTCAcagTGACCT) or a mutant ERE containing a 2-bp inversion (AGGTCAcagTG**CA**CT). Panel A, CHO-ER nuclear extract (NE) (lanes 1–3), E-Seph eluate (lanes 4–6), E-Seph eluate + p48/p45 (B-ERE→E-Seph nonadsorbed [NA]; lanes 7–9). Panel B, B-ERE eluate (lanes 1–3), B-ERE→E-Seph eluate (lanes 4–6), NA (lanes 7–9), B-ERE→E-Seph eluate + NA (lanes 10–12). Panel C, E-Seph/ATP eluate (lanes 1–3), E-Seph/ATP eluate + NA (lanes 4–6), E-Seph/ATP eluate + hsp70 (lanes 7–9), E-Seph/ATP eluate + NA + hsp70 (lanes 10–12).

presence of hsp70 and p55, in addition to hER (Fig. 2). Two additional proteins, p48 and p45, appear to be preferentially retained by DNA-affinity chromatography. Hsp70 has previously been shown to associate with GR and PR (19). The other three proteins have not been previously recognized as receptor-associated proteins. A virtually identical pattern of ER-associated proteins was observed by SDS-PAGE analysis of affinity-purified whole cell extract from MCF-7 (data not shown), indicating that the four copurified proteins obtained from CHO-ER cell extracts are not specific to CHO-ER cells, and that the presence of an artifactual variant of hER (Gly400Val) (27) in these cells is not a limitation. Furthermore, extracts of CHO-k1 cells, which lack ER, were used to demonstrate that significant retention of the proteins shown in Fig. 2 occurred only in the presence of hER. Therefore, we believe that we have a suitable model system for the isolation and characterization of ER-associated proteins.

Although SDS-PAGE silver stain analysis of the purified fractions allows for an estimate of the apparent molar ratios of the proteins present in isolated hER complexes, true stoichiometries are not known. To a first approximation, hsp70 appears to be present at a 1:1 molar ratio when hER is isolated by all three chromatographic approaches (Fig. 2, lanes 2–4). Treatment of the extract with ATP before purification leads to a selective reduction in ER-associated hsp70 following E-Seph chromatography (Fig. 2, compare lanes 1 and 2). The ratio of p55 to hER appears to vary according to the method of isolation (Fig. 2). p55 is clearly present in excess (2- to 4-fold) when hER is isolated by E-Seph chromatography, whereas much reduced levels of p55 were observed when hER was isolated by B-ERE and H222-Seph chromatography. The significance of this variability is unclear at this time. As shown in Fig. 2 (lane 3), p48 and p45 appear to be present at the lowest molar ratio to hER. However, their relative abundance and affinity for hER are unknown. Clearly, the observed stoichiometries can reflect a number of variables, including the abundance of each molecule, their affinity for hER and/or each other, the influence of modifying agents (*e.g.* ATP), as well as the silver staining properties of each protein. Whether the stoichiometries observed here reflect what occurs *in vivo* remains unknown. Therefore, any conclusions regarding the functional significance of the observed stoichiometries are speculative at this time.

p55, observed by all three chromatographic techniques (Fig. 2), was isolated by E-Seph chromatography and subsequently identified by N-terminal amino acid microsequencing as a protein disulfide isomerase (28). This protein was also independently identified by Cheng *et al.* (29) as a human cellular thyroid hormone binding protein. Although this result raises the possibility that p55 might associate with estradiol, our results do not

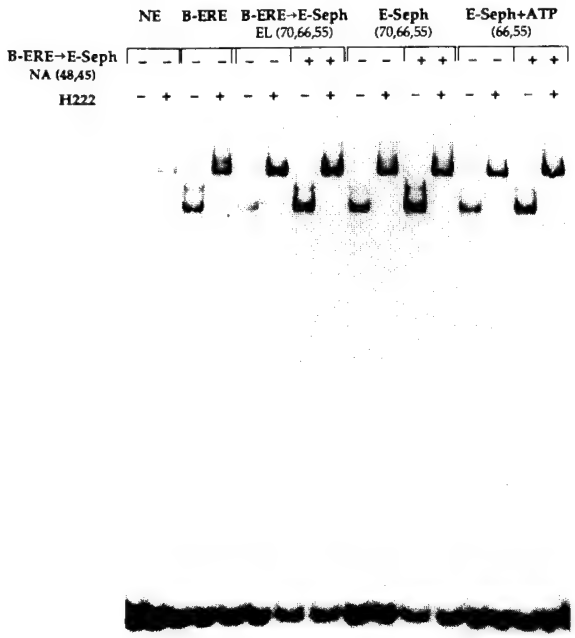

Fig. 7. Antibody Supershift Analysis of hER/^{32}P-ERE Interactions in the Presence or Absence of hER-Associated Proteins

hER complexes were incubated for 30 min at 25 C with ^{32}P-labeled ERE and electrophoresed on a 5% nondenaturing polyacrylamide gel. The source and composition of each mixture are outlined in Fig. 1. The B-ERE→E-Seph NA fraction (p48/p45) was added to hER complexes lacking p48 and p45 as indicated (+). H222, a monoclonal antibody directed against ER, was added as indicated (+) to demonstrate the presence of hER in the retarded complexes.

support this hypothesis. PDI has been studied in a variety of model systems and appears to participate in diverse activities (30). By indirect immunofluorescence and immunoelectron microscopy, p55 has been localized to the endoplasmic reticulum and the nuclear envelope (31, 32). As a homodimer, PDI has been described as a thioredoxin-like protein, catalyzing the isomerization of intramolecular disulfide bonds (33). The $\alpha_2\beta_2$ prolyl-4-hydroxylase tetramer contains PDI as the two β-subunits (34, 35). PDI also appears to be a part of the microsomal triglyceride transfer protein complex and the glycosylation site binding protein (36). In addition, PDI shows dehydroascorbate reductase activity (37). The contribution of PDI, and especially its catalytic activity, to these different complexes remains unclear.

Recent mutational analyses and complementation studies in yeast have attempted to address the functional significance of PDI. Complete knockout of PDI results in a lethal phenotype, clearly demonstrating that PDI is an essential protein for yeast viability (38). However, when one or both active sites are deleted from the catalytic domain, cells display normal growth patterns. These data suggest that the essential function of PDI does not reside in its catalytic properties (39). This conclusion is further supported by the finding that Eug1, a natural yeast PDI homolog that lacks catalytic activity, can sustain cell viability in PDI knockout strains. Additional deletions in the C-terminal 152 amino acids of PDI, which removes the ER-retention signal, also do not affect cell growth. These studies strongly suggest that PDI has essential roles outside of the enzymatic activities observed in the endoplasmic reticulum-resident PDI population.

An interesting property of PDI that distinguishes it from the other thioredoxin-like molecules is its ability to bind peptides (40). Modifications of the active site cysteines that block isomerization activity do not affect PDI peptide binding. However, peptide binding to PDI appears to inhibit PDI-catalyzed protein folding of bovine pancreatic trypsin inhibitor, and the extent of inhibition is proportional to the amount of peptide bound (40). The peptide binding domain of PDI maps to residues 451–476 in the highly acidic C-terminal region. The significance of this peptide binding activity of PDI remains unclear, as does PDI's essential *in vivo* function. It has been suggested that PDI may act as a molecular chaperone, much like PPI or members of the hsp family (41, 42). However, the contribution of PDI to estrogen receptor function and the significance of their association await further characterization.

Two additional proteins, p48 and p45, were observed in eluates containing hER isolated by B-ERE chromatography (Fig. 2). Although two proteins of a similar mol wt were also present in some H222-Seph eluates, the intensity of the p48 and p45 bands was enhanced when hER was bound to an ERE, suggesting that their association with hER is promoted or stabilized by ER-ERE interaction. The identity of p48 and p45 remains unknown, but is being investigated by the approach used to identify p55. A 45-kDa single stranded DNA-binding protein has been reported to be necessary for high affinity binding of ER to the vitellogenin A2 ERE (43). This protein, although a single stranded DNA binding protein, could not be substituted for by the purified *E. coli* SSB, suggesting that the effect is not a general property of single stranded binding proteins. It is possible that one of the proteins we have detected could be this single stranded DNA binding protein.

The nature of agonist- *vs*. antagonist-receptor interaction and the resulting altered transcriptional activity is still poorly understood. It is possible that an altered conformation of receptor occurs in the presence of an antagonist which could affect receptor stability, DNA binding, interaction with other transcription factors, phosphorylation, or interaction with hsp90 or hsp70. It has recently been observed that the carboxy-terminal tail of several steroid receptors may be a critical determinant of receptor response to ligand and that antagonism may result from a failure to interact with a 30-amino acid domain at the carboxy terminus (44). For the complete estrogen antagonist, ICI-164 (or ICI-182), it has been suggested that interference with receptor dimerization (45) or promotion of receptor degradation (46) may occur. The mechanism by which the partial agonist, OH-Tam, allows transcriptional activity from some promoters but inhibits activity from others (47) remains unclear. In light of the importance of under-

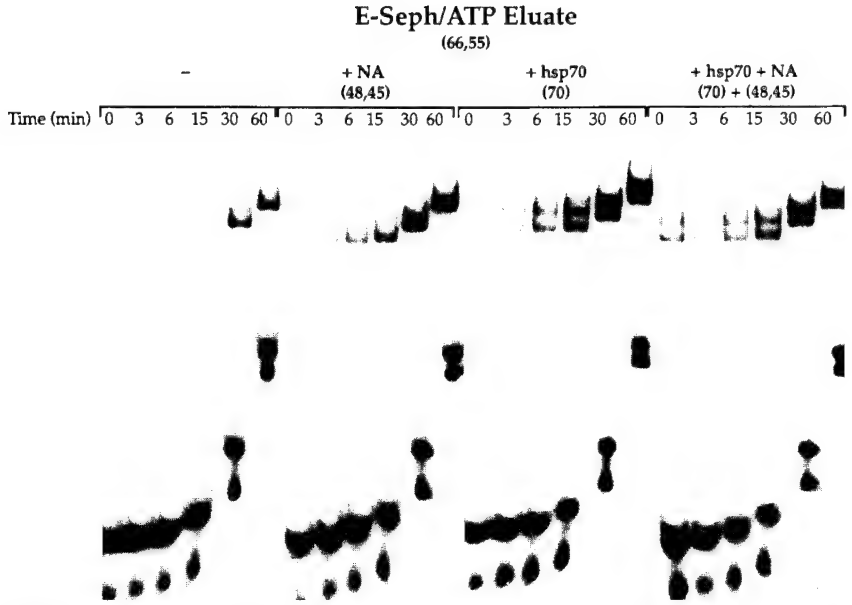

Fig. 8. Analysis of hER/^{32}P-ERE Interactions in the Presence or Absence of hER-Associated Proteins to Determine the Rate of ER-ERE Association

hER complexes were incubated for 0–60 min at 25 C with ^{32}P-labeled ERE. At the indicated time points, samples were loaded directly onto a running 5% nondenaturing polyacrylamide gel. The source and composition of each mixture are outlined in Fig. 1. A 60-min time course was performed for each of the following mixtures: E-Seph/ATP (hER/p55), E-Seph/ATP + NA (p48/p45), E-Seph/ATP + hsp70, and E-Seph/ATP + NA + hsp70.

standing receptor-antagonist interaction we chose to investigate the possible differential effects of agonists and antagonists *in situ* on the interaction of hER with the observed associated proteins.

CHO-ER cells were metabolically labeled with [^{35}S]-methionine during treatment with estradiol, ICI-182, or OH-Tam, and hER complexes were subsequently isolated by H222-Seph or B-ERE chromatography (Figs. 4 and 5). None of the tested ligands had an effect on the stoichiometry of protein association with hER isolated by DNA chromatography. However, when ER complexes were isolated by H222-Seph chromatography, a significant reduction in the amount of associated hsp70 was observed following treatment of CHO-ER cells *in situ* with estradiol or the partial antagonist OH-Tam, whereas dissociation of hsp70 did not occur in the absence of ligand or when cells were treated with ICI-182. In contrast to other published reports on the effects of ICI-164 (an analog of ICI-182) on ER stability in mouse uterus (46), no significant loss of ER was observed in extracts of CHO-ER cells that had been treated *in situ* with ICI-182.

The purification of hER complexes by immunoadsorption on H222-Seph affords an 80–90% recovery of total cellular receptor, whereas B-ERE chromatography yields only a 30–50% recovery of hER. We believe these data demonstrate that the immunoadsorbent is able to isolate total cellular receptor complexes, whereas B-ERE chromatography can isolate only that portion of the receptor population that is competent for DNA binding. The metabolic labeling experiments show that *in vivo* treatment with estradiol or OH-Tam weakens the hER-hsp70 interaction. Our ability to isolate hsp70-hER complex *in vitro* by E-Seph or H222-Seph chromatography in the absence of ATP may indicate that the ligand-mediated *in vivo* mechanism for destabilizing the hsp70-ER interaction involves ATP or an ATP-regenerating system, as proposed for PR by Smith and Toft (2, 48, 49). The results of the metabolic labeling experiments further suggest that in the absence of specific DNA during isolation, the hsp70-hER interaction is lost or significantly weakened, whereas when specific DNA is present, the interaction is stabilized. It is possible that ER-DNA binding proceeds until all necessary regulatory sites are occupied. Once this is accomplished, without the availability of specific DNA sites, the hsp70-hER interaction is lost, terminating the activation signal. It is also possible that removal of hsp70 from the DNA bound complex may destabilize the interaction of receptor with DNA, thereby terminating regulation from that responsive site. The results observed when cells were treated with the complete estrogen antagonist ICI-182 suggest that ICI-bound receptor does not promote a conformation that weakens the hsp70-hER interaction. In contrast, OH-Tam-bound hER appears to participate in at least some protein and DNA interactions that mimic estradiol-bound receptor, suggesting that the antagonist effects of OH-Tam occur after the DNA binding event.

The electrophoretic mobility shift assay was used to examine the influence of the ER-associated proteins on the affinity and/or rate of ER-ERE complex formation. Both removal of ER-associated components and reconstitution experiments were used to address this question (Fig. 6). As described in the results, maximal interaction of hER with the vitellogenin ERE occurred in the

presence of all four hER-associated proteins isolated by B-ERE chromatography (hsp70, hER, p55, p48, and p45). This interaction was as stable as that observed for hER in crude nuclear extracts. Notably, the B-ERE eluate gives rise to two hER-ERE complexes. Removal of p48 and p45 afforded a [^{32}P]ERE/hER complex of significantly reduced intensity. As mentioned earlier, the p48/45 fraction alone did not interact with the ERE.

A minimal hER complex was generated by treating CHO-ER whole cell extracts with ATP before purification by steroid-affinity chromatography (Fig. 1). The resulting hER/p55 complex displayed the weakest detectable interaction with the ERE (Fig. 6). Thus, hsp70 may play a role in stabilizing or facilitating ER-ERE interaction. It has been reported that hsp70 is not present or involved in binding of progesterone receptor to its response element (50). However, recent experiments have shown that baculovirus overexpressed human GR is associated with hsp70 when bound to a glucocorticoid response element (10). Therefore, ER may function differently than PR, and more like GR, with respect to the hsp70 interaction. Thus, it will be especially important to further characterize the role of hsp70 in ER-mediated transcriptional activation or in the stabilization of ER-ERE interactions.

Although reconstitution of dissociated receptor-protein complexes has been reported to be problematic for the progesterone receptor (49), the data presented here indicate that reconstitution of hER complexes is possible. Stoichiometric mixtures (relative to their initial purification) of either the p48/p45 fraction or purified hsp70 were added to the E-Seph/ATP complex to attempt reconstitution of a more stable hER-ERE complex (Fig. 6). As described in the results, addition of the p48/p45 fraction to the E-Seph/ATP complex led to a marked enhancement in the [^{32}P]ERE/hER interaction. Hsp70, when added separately to this E-Seph/ATP complex, resulted in a similar stabilization of the [^{32}P]-ERE/hER interaction as well as the generation of a second, more slowly migrating, complex. The most intense [^{32}P]ERE/hER interaction was detected when both the p48/p45 fraction and hsp70 were added to the E-Seph/ATP complex, comparable to the DNA-affinity purified material containing the same proteins. These results suggest that maximal interaction of the hER with the vitellogenin ERE requires either p48, p45, or both, in addition to hsp70. The contributions of the remaining hER-associated proteins will be examined as they are purified. Finally, our data demonstrate that reconstitution of dissociated hER-complexes is indeed possible, at least for some *in vitro* associated proteins.

The significance of the gel shift doublet observed for hER purified by B-ERE (Fig. 6B, lane 1) is unclear. hER was shown by antibody supershift experiments to be present in both complexes (Fig. 7). Clearly, the relative ratio of hER to other cellular proteins has been altered and therefore other intermediates of hER complexes may be visible that are not favored in crude extracts. The doublet observed in the B-ERE fraction was diminished when the p48/45 fraction was removed, suggesting that one or both of these components are a part of the more slowly migrating complex (Fig. 6). Addition of the p48/45 fraction to complexes lacking these proteins generates a very weak doublet which may simply indicate that our reconstitution is not 100% efficient. The E-Seph fraction (hsp70, hER, p55) also shows a weak doublet which reverts to a singlet upon removal of hsp70. These results suggest that hsp70 may be a component of the upper complex. This hypothesis is strengthened by the observation that addition of purified hsp70 to the E-Seph/ATP complex (hER, p55) generates a doublet. Furthermore, addition of hsp70 and the p48/p45 fraction to the E-Seph/ATP complex generates the doublet plus a third, less intense, but detectable complex.

A final set of experiments was performed to examine the rate of ER-ERE interaction in the presence of different hER-associated proteins (Fig. 8). Gel shift reactions were prepared as described, and aliquots were removed from the reactions over time and immediately loaded on a running gel. The slowest rate of complex assembly with the [^{32}P]ERE was observed for the hER/p55 (E-Seph/ATP) complex (Fig. 8). The separate addition of either hsp70 or the p48/p45 fraction contributed to a significantly enhanced rate of association. The maximum association rate occurred in the presence of all four hER-associated proteins (Fig. 8). These additional reconstitution studies indicate that a major contribution of these ER-associated proteins to hER-ERE interaction is to enhance the rate of association.

Based on the data reported here and the published data of others regarding receptor-associated proteins, we propose the model shown in Fig. 9. In this model, unactivated ER-p55-hsp70/90 complex loses hsp90 following ligand binding. The resulting activated ER complex recruits or stabilizes the binding of two additional proteins, p48 and p45, when hER binds to ERE.

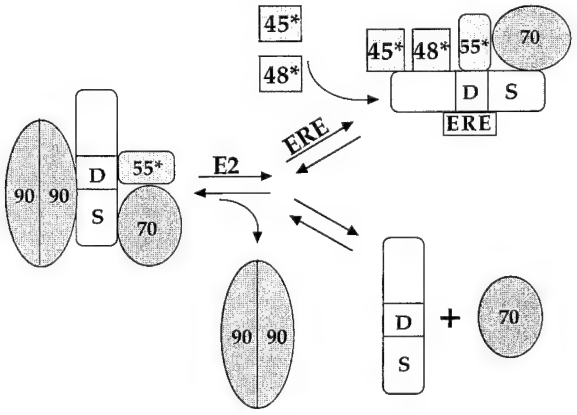

Fig. 9. Model for hER-Associated Proteins

This model is based on our preliminary data and data reported by others for ER, PR, and GR. Contact sites between hER and p45, and between p48 and p55 are unknown (indicated by *). Also, interactions among associated proteins are not resolved. D, DNA binding domain; S, steroid binding domain.

The population of liganded hER that does not bind to ERE dissociates from hsp70. Other proteins (*i.e.* hsp56) that are not shown may participate in one or more of these steps. The interaction of ER with components of the transcription initiation complex (*e.g.* TFIIB) are not shown. This model is not meant to be complete. Future work will focus on the identification and further characterization of these, and other, ER-associated proteins, especially in regard to their ability to influence DNA binding and/or transcriptional activation by ER.

MATERIALS AND METHODS

Materials

[6,7-^{3}H]Estradiol and [^{35}S]methionine were obtained from Amersham Life Science (Arlington Heights, IL). [γ-^{32}P]ATP, crude, was obtained from ICN Biomedicals (Irvine, CA). Dulbecco's modified Eagle medium/Ham F-12 nutrient mixture (1:1) without phenol red, Seru-Max-4, an iron-supplemented calf serum, aprotinin, leupeptin, and BNPS-Skatole [3-bromo-3-methyl-2-(nitrophenylmercapto)-^{3}H-indole] were supplied by Sigma (St. Louis, MO). Pefabloc-SC was purchased from Pentapharm AG (Basel, Switzerland). PBS for cell culture was purchased from Gibco-BRL (Grand Island, NY). Amino-hexanoyl-biotin-N-hydroxysuccinimide ester for biotinylation of amino-linked oligonucleotides was purchased from Zymed Laboratories (San Francisco, CA). UltraAvidin-Agarose for DNA-affinity chromatography was supplied by Leinco Technologies (St. Louis, MO). CNBr-activated Sepharose 4B and thiopropyl-Sepharose 6B used in the construction of the immunoadsorbent and estradiol column, respectively, were purchased from Pharmacia (Piscataway, NJ). Poly(dIdC) was also purchased from Pharmacia. ICI-182,780 and OH-Tam were kindly provided by Alan Wakeling at ICI Pharmaceuticals. T_4 polynucleotide kinase was purchased from United States Biochemical (Cleveland, OH). Monoclonal antibody H222 was prepared against purified human ER (51), monoclonal antibody hsp70.7.10 directed against hsp70 was prepared by S. L. Lindquist (52), polyclonal antibodies to p55 were prepared by S. Y. Cheng (32), and an additional peptide antibody, yielding identical results, was prepared in our laboratory (unpublished results). Rabbit anti-rat IgG, rabbit anti-mouse IgG, biotinylated goat anti-rabbit IgG, horse radish peroxidase (HRP)-Protein A, and HRP-Streptavidin were all purchased from Zymed Laboratories (San Francisco, CA). Chemicals for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Melville, NY). Immobilon-P transfer membrane was purchased from Millipore (Bedford, MA). CPGs (500 Å pore size) were obtained from Electronucleonics (Fairfield, NJ). Protein standards for mol wt determinations were from Bio-Rad.

Preparation of CHO-ER Whole Cell or Nuclear Extracts

CHO-ER cells (18) were cultured in Dulbecco's modified Eagle medium/Ham F-12 Nutrient Mixture (1:1) without phenol red, supplemented with 10% iron-supplemented newborn calf serum that did not require charcoal treatment. To maintain expression and selection of the ER gene, 50 μM $ZnSO_4$ and 40 μM $CdSO_4$ were also included in the medium. Confluent cells were harvested by treatment with trypsin, followed by extensive washing with PBS and pelleted by centrifugation at 1000 × *g*. For whole cell extraction, the cell pellet was resuspended in 4 vol ice-cold extraction buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 400 mM NaCl, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.2 mM PEFABLOC). Cells were lysed in an ice bath with a dounce or polytron homogenizer and extracted for 20 min. The lysed cells were pelleted at 4 C by centrifugation at 10,000 × *g* and the supernatant was frozen in aliquots and stored at −70 C. ER content was determined by the CPG assay as previously described (25) after treatment of the extract with excess [6,7-^{3}H]estradiol.

For the preparation of nuclear ER, cells in culture were treated with 10 nM [6,7-^{3}H]estradiol for 1 h at 37 C before harvesting. The cell pellet was resuspended in a hypotonic buffer (extraction buffer without NaCl) and homogenized as described above. The nuclei were pelleted and extracted in a buffer that contained 400 mM NaCl. ER content was measured by CPG analysis.

Biotinylation of Amino-Linked Oligonucleotides

A 27-bp oligonucleotide containing the estrogen response element (ERE) from the vitellogenin A2 gene (GATCCTAGAGGTCACAGTGACCTACGA) (53) was synthesized with a 6-carbon spacer and a terminal amino group (Genetic Designs; Houston, TX). Ten nanomoles of the oligo were added to 0.1 M $NaHCO_3$, pH 9.1, in a final volume of 50 μl. Two milligrams of amino-hexanoyl-biotin-N-hydroxysuccinimide ester were dissolved in 25 μl N,N-dimethylformamide and added, with vortexing, to the oligo solution and incubated for 90 min at 40 C; 125 μl water, 20 μl 3 M sodium acetate, and 800 μl absolute ethanol were added to the mixture. After a 30 min incubation at −70 C, the oligo was pelleted by centrifugation at 10,000 × *g* for 15 min. The pellet was washed with 70% ethanol and dried in a speed-vac. The dried pellet was resuspended in 50 μl water and mixed with an equal molar quantity of the complementary strand. TE buffer containing 0.2 M NaCl was added to yield a final volume of 100 μl. The mixture was heated to 95 C for 5 min in a heating block and allowed to cool slowly to room temperature (4 h). Duplex DNA was precipitated and dried as described above.

Purification of hER from CHO-ER Extracts

Estradiol-Sepharose Chromatography CHO-ER whole cell extract (2.5 ml), adjusted to contain 0.7 M NaCl and 1 M urea, was applied to a 200 μl estradiol-Sepharose column (20) and incubated batchwise for 1 h at 4 C. The column was washed with 20 bed volumes each of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 700 mM NaCl, 1 M urea), and the same buffer with 400 mM NaCl and 3 M urea. Bound ER was eluted with 2 × 10^{-5} M [6,7-^{3}H]estradiol in a buffer that contained 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 200 mM NaCl, and 5 M urea. The yield of ER was determined by specific adsorption to CPGs.

H222-Immunoaffinity Chromatography CHO-ER whole cell extract (2.5 ml) was adjusted to contain 1 M urea and labeled with excess [6,7-^{3}H]estradiol for 1 h at 4 C. The extract was applied to 200 μl H222-Sepharose beads (25) in a 10 ml Bio-Rad Econo Column and incubated batchwise for 1 h at 4 C. The beads were then washed with 20 bed volumes of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 400 mM NaCl, 1 M urea). Bound ER was eluted in a buffer containing 50 mM Tris, pH 7.4, 2 M NaSCN, 10% N,N-dimethylformamide at 22 C. The yield of ER was determined both by CPG assay as well as by direct counting in scintillation cocktail.

DNA-Affinity Chromatography CHO-ER whole cell extract (2.5 ml) was labeled with excess [6,7-^{3}H]estradiol for 1 h at 4 C and then dialyzed against a buffer containing 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, and 1 M urea. ER content in the extract was determined by CPG assay. Excess biotinylated ERE (B-ERE) was added to the extract at a ratio of 5 pmol ERE to 1 pmol ER along with 50 μg poly(dIdC) and 10 μg progesterone response element from the MMTV long terminal repeats (TGACTTGGTTTGGTACAAAATGTTCT GATCTG) as carrier DNA. This mixture was incubated for 20 min at 22 C, followed by an additional incubation for 40 min at 4 C, and applied to a 200 μl UltraAvidin-agarose column and incubated batchwise for 1 h at 4 C. The column was washed

with 20 bed volumes of loading buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1 M urea). Bound ER was eluted in a buffer containing 20 mM Tris, 1 mM EDTA, 1 mM DTT, 1 M NaCl, and 1 M urea and quantitated by CPG assay as well as by direct counting in scintillation cocktail.

For the isolation of the 48- and 45-kDa proteins, unlabeled CHO-ER whole cell extract was eluted from the B-ERE/Streptavidin-agarose beads in a buffer containing 700 mM NaCl and applied to the estradiol-sepharose column. Subsequent chromatography on the estradiol-Sepharose column proceeded as described above.

When CHO-ER nuclear extracts were used the procedure was the same as described except that incubation with [6,7-^{3}H]estradiol was not necessary since the cells were prelabeled with [6,7-^{3}H]estradiol in culture.

SDS-PAGE and Staining

Partially purified protein samples prepared by the described chromatographic techniques were analyzed by separation on reducing 10% SDS-polyacrylamide gels (12.5 × 14 × 1.5) containing 0.39% N,N′-diallyltartardiamide, 0.375 M Tris, pH 8.8, 0.1% SDS, 0.035% ammonium persulfate, and 0.025% N,N,N′,N′-tetramethylethylenediamine. Before electrophoresis, samples were diluted in sample buffer (50 mM Tris, pH 6.8, 10% sucrose, 2% SDS, 5% β-mercaptoethanol, and 0.005% bromophenol blue) and heated at 95 C for 5 min. Aliquots of each sample were applied to the wells of a 7% polyacrylamide stacking gel and electrophoresis was carried out at 4 C overnight at 10 mA.

For Western blot analysis, separated proteins were transferred electrophoretically from the gel to nitrocellulose by a modified method of Towbin *et al.* (54). Electrophoretic transfer was carried out at 4 C for 2 h at 1.0 A in a buffer containing 0.025 M glycine, 0.192 M Tris, and 10% methanol. Following transfer, the nitrocellulose was stained with Ponceau S (Sigma), and lanes containing mol wt markers were cut out. The nitrocellulose blots were treated with 3% Carnation nonfat dry milk in Tris-buffered saline, pH 7.4, containing 0.02% Tween 20 (TBS-Tween) for 1 h to saturate the nitrocellulose with protein. A second incubation was carried out with monoclonal antibody to ER (4 μg/ml) (20), hsp70 (1:2000) (52), or p55 (1:2000) (32) in 1% milk in TBS-Tween for 1 h at 22 C. Incubation of the blots for an additional hour was performed with 2 μg/ml of either rabbit anti-rat immunoglobulin G (IgG) (ER), rabbit anti-mouse IgG (hsp70, p55), or biotinylated goat anti-rabbit IgG (p55) before a final 1 h incubation in a 1:1000 dilution of either HRP-Protein A (ER, hsp70, p55) or HRP-Streptavidin (p55). After each incubation, blots were washed with TBS-Tween. Proteins were visualized by incubation with 5 mg diaminobenzidine in Tris-buffered saline, pH 7.4, in the presence of 30 μl 30% H_2O_2.

For silver stain analysis, gels were fixed in isopropanol-acetic acid-water (2:1:8), followed by 10% glutaraldehyde. After extensive washing (3–5 h) with distilled water, gels were stained with silver as described previously (25).

Digestion of the 55-kDa Protein with BNPS-Skatole

Proteins present in the hER complex eluted from the E-Seph column were separated by SDS-PAGE and transferred to Immobilon-P as described above. After staining with Ponceau S, the 55-kDa band was excised, cut into small pieces, and placed in a microfuge tube. BNPS-Skatole (1 μg/μl) in 75% acetic acid was added and the tube was incubated at 47 C for 1 h (21). The liquid was removed and the membrane was washed thoroughly with water to remove the acetic acid. The washed membrane was dried by vacuum centrifugation and incubated in a buffer containing 50 mM Tris, pH 9.1, 2% SDS, 1% Triton X-100 for 2 h at room temperature to elute the fragments. An appropriate volume of a 6× solution (60% sucrose, 100 mM DTT, and 0.3% bromophenol blue) of diluent was added to the mixture and the incubation was continued for an additional hour. The eluted fragments were then separated by SDS-PAGE and transferred to Immobilon-P. Transferred fragments were visualized by staining with Coomassie brilliant blue R-250, and selected fragments were excised and sent to the University of Kentucky Macromolecular Structure Analysis Facility for sequencing. The results are as follows. CHO-ER fragment 1: APEEEDNVLVLKKSNFKEALAA; CHO-ER fragment 2: NYLLVEFYA; CHO-ER fragment 3: LAKQTGPAATTL; MCF-7 fragment 1: APEEEDHVLVLRKSN FA.

^{35}S-Methionine Metabolic Labeling of CHO-ER cells

CHO-ER cells were seeded into six-well plates in complete media and incubated as described earlier. The media were removed 12–18 h later and the cells were washed 2 × 2 ml with PBS. The cells were then incubated in methionine-free media for 1 h at 37 C in a humidified, 5% CO_2 atmosphere. These media were removed and replaced with [^{35}S]methionine-containing media (0.1 mCi/well) in the presence of either ethanol vehicle, 10 nM estradiol, 100 nM ICI 182,780, or 100 nM OH-Tam and incubated for 2 h. Cells were released with trypsin, washed thoroughly with PBS, and lysed by freeze-thaw in a buffer containing 10 mM Tris, 1 mM EDTA, 1 mM DTT, 400 mM NaCl, 1 M urea, and protease inhibitors (aprotinin, leupeptin, and PEFABLOC). The mixture was passed through a 25 gauge needle to sheer the DNA and clarified by centrifugation at 10,000 × g at 4 C for 20 min. The supernatant was collected and the protein concentration was determined by a modified Coomassie blue assay (55). Samples were standardized for total protein concentration and hER complexes were subsequently isolated by either H222-Seph or B-ERE chromatography.

Electrophoretic Mobility Shift Assay

Forty nanograms of a 27-bp, double stranded, synthetic oligonucleotide, corresponding to the vitellogenin A2 ERE (same sequence as described above), was end-labeled with 200 μCi [γ-^{32}P]-dATP using 5–8 U T_4 polynucleotide kinase (56). After radiolabeling, the DNA was extracted with phenol-chloroform, precipitated with ethanol, and dried as described earlier. The pelleted DNA was resuspended in a buffer containing 20% glycerol, 25 mM HEPES, 1.0 mM EDTA, 1.0 mM DTT, and 70 mM NaCl. The DNA was then resolved on a 9% nondenaturing polyacrylamide gel (1× TBE). The gel was exposed to film to visualize the position of the duplex DNA, which was subsequently excised. The gel slice was crushed using a glass rod and extracted for 2 h at 37 C in a buffer containing 0.5 M sodium acetate, pH 5.0, and 1.0 mM EDTA. The eluted DNA present in the supernatant was extracted with phenol-chloroform, precipitated with ethanol, and dried as described above.

Ten to 25 fmol ER (determined by specific adsorption to CPGs) were incubated with 20–45 fmol (8,000–12,000 cpm) of [^{32}P]-ERE for 30 min at 22 C. All incubations were performed in the presence of 1 μg poly(dIdC), 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 20% glycerol. The total protein concentration in all incubations was normalized to 5 μg by the addition of BSA when necessary. The samples were analyzed on a nondenaturing, 5% polyacrylamide gel (0.5× TBE) for 2 h at 10 mA. Radioactive bands were visualized on dried gels by autoradiography at −75 C with chromex intensifying screens for 3–12 h.

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